QUANTITATIVE AND CYTOCHEMICAL STUDIES OF HUMAN GINGIVAL CREVICULAR NEUTROPHILS: MYELOPEROXIDASE ACTIVITY AND NITROBLUE TETRAZOLIUM REDUCTION

by

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Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

1984

.... Then felt I like some watcher of the skies
When a new planet swims into his ken
Or like stout Cortez when with eagle eyes
He star'd at the Pacific - and all his men
Look'd at each other with a wild surmise -
Silent, upon a peak in Darien.

(Keats, 1816)

The investigations and procedures described in this thesis were designed and performed by the author, except where specifically stated as otherwise in the acknowledgements.

No portion of this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institution of learning.

Contents

Abstract

Neutrophil leucocytes constitute the principal cellular defence against microorganisms and are essential for life. Neutrophils enter the oral cavity predominantly via the gingival crevice, where little is known of their function. Phagocytic and microbicidal activity of gingival crevicular neutrophils has been reported in limited studies and the purpose of the present investigation was to extend the study of neutrophils collected from the gingival crevice.

The high proportion of neutrophils found in the crevice confirmed previous work. Many contained intracellular bacteria and the majority stained positively for alkaline phosphatase. More detailed studies were undertaken of two main components of the oxygen-dependent microbicidal system.

Nitroblue tetrazolium (NBT) dye reduction was used as an indirect assay of neutrophil metabolism and superoxide anion production, and the enzyme myeloperoxidase (MPO) was assayed as a specific factor contributing to microbicidal activity. Crevicular neutrophils consistently showed significantly higher NBT reduction than neutrophils from peripheral blood, illustrating their ability to respond to local bacterial stimuli. There was a positive correlation between blood and crevicular neutrophil values for NBT reduction, which may

therefore provide a predictive assay of crevicular neutrophil function.

Nitroblue tetrazolium was used as a marker to isolate the reducing activity of whole saliva. This activity was located in the bacterial fraction, so that there is a need for caution when bacteria are used as cell stimulants in NBT tests.

Myeloperoxidase was present in the crevicular neutrophils of all subjects examined but enzyme activity was variable, being absent in one study group. It is possible that neutrophils migrate selectively into the gingival crevice. There was a positive correlation between the degree of gingival inflammation measured clinically and MPO activity. Also, the crevicular enzyme was shown to be altered by the in vivo administration of antibiotics, significantly more so than the blood enzyme in the case of cefaclor, a chlorinated cephalosporin.

When the NBT and MPO assays were applied to crevicular cells from patients with neutrophil dysfunction (neutropenia, leukaemia and chronic granulomatous disease), their sensitivity to both disease and treatment was apparent.

Studies of crevicular neutrophils may therefore provide information relevant to understanding the aetiology and pathogenesis of periodontal disease, and

may also provide an accessible source of neutrophils by which to monitor host defence mechanisms in patients with disorders of neutrophil production and function.

Acknowledgements

These studies were made possible by the generous help and cooperation of many people.

In addition, financial support was provided by the Scottish Home and Health Department. The Myeloperoxidase studies were made possible by Lily Industries, USA, whose generosity together with the British Council, also helped to finance a visit to Professor Robert Baehner, Indianapolis, USA.

Torquil MacPhee first stimulated my interest in neutrophil function related to periodontal disease and has since provided every encouragement. Howard Moody and Peter Newman were tolerant of my inexperience and gave invaluable advice during the early stages of this work. I am grateful to Pamela Galloway, Jean Naysmith, Yvonne Crombie and Ronnie Sutherland who all at various times helped with staining the many glass slide preparations. Chris Cumming gave expert advice on bacteriological methods and Dr David Reeves of Bristol performed the original serum cefaclor assays.

I am indebted to all the students and colleagues who so patiently provided samples of blood and crevicular fluid, and Drs Norman Allan and George Stockdill for their enthusiastic cooperation and for allowing me to study patients under their care. Professor Robert Baehner, Drs Larry Boxer (Indianapolis) and particularly

Anna-Maija Raeste (Helsinki) provided both hospitality and invaluable constructive criticism of my ideas. Statistical advice was patiently given by Robin Prescott and Jeffrey Sofaer. Frank Collier and Ian Lennox executed the line drawings and Ian Goddard produced the photographs with typical efficiency.

I am extremely grateful to my sister, Danusia Smallman for translating the Japanese article by Dr Kusunoki and to my father for translating the French, German and Spanish papers. Mr John Mitchell and my wife, Joan, kindly proof-read the manuscript and May Norquay exercised considerable secretarial skill throughout all stages of development and production. Particular thanks go to Marjory Grant who so cheerfully lent her biochemical expertise and assistance to the spectrophotometric assays of myeloperoxidase. I am indebted to Professor John Southam for his friendly supervision and advice.

Sandy Raeburn devoted many hours to discussing the experimental work at all stages and generously made the necessary laboratory facilities available. In expressing my gratitude, I am also pleased to say that, since our original meeting over one of his patients in an operating theatre, a firm friendship has developed.

Finally, and above all, I owe an enormous debt of gratitude to Joan for her continual optimism, tolerance, encouragement and unwavering support without which this study would never have reached completion.

Part of the work presented in this thesis has been published in the following papers:

1. Kowolik, M.J. and Moody, G.H. 1979 A simplified technique for the NBT test. *IRCS Medical Science. Key Reports in Cell and Molecular Biology,* 7: 100.
2. Kowolik, M.J. and Raeburn, J.A. 1980 Functional integrity of gingival crevicular polymorphonuclear leukocytes as demonstrated by NBT reduction. *Journal of Periodontal Research,* 15: 483-491.
3. Kowolik, M.J. 1982 NBT positive bacteria in human saliva: a possible artefact in NBT tests. *Journal of Infection,* 4: 215-219.
4. Kowolik, M.J., Raeburn, J.A. and Grant, M. 1982 *In vivo* effect of ampicillin and cefaclor on blood and gingival crevicular neutrophil myeloperoxidase activity. In: *The Influence of Antibiotics on the Host-Parasite Relationship.* Eds. Eickenberg, H.-U., Hahn, H. and Opferkuch, W. Springer-Verlag, Heidelberg, pp.56-66.
5. Kowolik, M.J. and Raeburn, J.A. 1983 NBT reduction by exudate neutrophils in carriers of chronic granulomatous disease. *Journal of Infection,* 6: 96-97.
6. Kowolik, M.J. and Grant, M. 1983 Myeloperoxidase activity in human gingival crevicular neutrophils. *Archives of Oral Biology,* 28: 293-295.

List of Abbreviations

ABTS	-	2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid)
ALL	-	acute lymphoblastic leukaemia
AML	-	acute myeloid leukaemia
AMMøL	-	acute myelomonocytic leukaemia
AMøL	-	acute monocytic leukaemia
ATP	-	adenosine triphosphate
BCG	-	Bacillus Calmette-Guérin
CGD	-	chronic granulomatous disease
CLL	-	chronic lymphocytic leukaemia
CML	-	chronic myeloid leukaemia
CSF	-	colony stimulating factor
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediaminetetraacetic acid
Eh	-	oxidation-reduction potential
FAD	-	flavin adenine dinucleotide
FMLP	-	N-formyl-methionyl-leucyl-phenylalanine
GI	-	Gingival Index
GSH	-	reduced glutathione
GSSH	-	oxidised glutathione
HLA	-	human leucocyte antigen
HMPS	-	hexosemonophosphate shunt
LPO	-	lactoperoxidase
MPO	-	myeloperoxidase
NAD	-	nicotinamide adenine dinucleotide
NADH	-	reduced nicotinamide adenine dinucleotide
NADP	-	nicotinamide adenine dinucleotide phosphate
NADPH	-	reduced nicotinamide adenine dinucleotide phosphate
NAP	-	neutrophil alkaline phosphatase
NBT	-	nitroblue tetrazolium
NT	-	neotetrazolium
OD	-	optical density
OMR	-	orogranulocyte migratory rate

OMRI	-	oral migratory rate index
PBS	-	phosphate buffered saline
PVB	-	peripheral venous blood
sds	-	saline dextrose solution
SE%	-	standard error of a percentage
sem	-	standard error of the mean
SOD	-	superoxide dismutase
TRAMPCOL	-	Regime of chemotherapy used in leukaemia, including thioguanine, daunorubicin, cytosine arabinoside, methotrexate, prednisolone cyclophosphamide, vincristine and L-asparaginase
WBC	-	white blood cell

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CHAPTER 1

GENERAL REVIEW AND INTRODUCTION

PHAGOCYTIC CELLS

Those mammalian cells regarded as committed phagocytes, and an integral part of normal host defence mechanisms, are the monocytes/macrophages and the neutrophil and eosinophil polymorphonuclear leucocytes. Many other cell types, such as fibroblasts and epithelial cells (Wolff and Konrad, 1972; Tripathi and Tripathi, 1982), have been shown to be capable of internalising particles or bacteria, but the essential distinction is the ability of committed phagocytes to respond rapidly to a bacterial stimulus, and following phagocytosis, to kill the organisms. Specialisation is shown by the increased efficiency of this system if the organisms have been opsonised with antibody or activated complement, for which the phagocytes have surface receptors. Other non-specific mechanisms also facilitate attachment. In the case of monocyte or macrophage activity, phagocytosis may be an integral part of its role in immunological, as opposed to strictly inflammatory, processes.

This vital protective response to external attack was first described and placed in biological perspective by the zoologist Metchnikoff 100 years ago. He observed wandering cells in starfish larvae, and how they localised round the site of an injected thorn

(Metchnikoff, O., 1918*). Subsequently he developed his theory of phagocytosis, although it is clear that much experimentation had already been undertaken (Metchnikoff, 1880; Karnovsky, 1981), and that the studies of other zoologists such as Haeckel (1862) provided additional inspiration. The first comprehensive account of this cellular defence mechanism, based particularly on experiments with Daphnia (Metchnikoff, 1884), was followed by recognition of its importance by others and a request for further information, and thus extended experimentation. An editorial footnote to a paper published by invitation in Annales de l'Institut Pasteur (Metschnikoff, 1887) noted: "A raison de l'intérêt et de l'attention qu'excite en ce moment la nouvelle théorie des phagocytes de M. Metschnikoff, nous avon demandé à l'auteur de vouloir bien l'exposer lui-même dans ces Annales".

Metchnikoff argued that phagocytosis as a protective phenomenon had developed in complex animals (from crustaceans to man) directly from the feeding mechanisms of protozoans and sponges. This was further emphasised by Hofmeister's work, demonstrating the presence of proteolytic enzymes, chemically similar, both in

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* Mme Olga Metchnikoff was the zoologist's wife, and her biography, originally published in 1918 in French, was translated into English by the family friend, Sir Roy Lankester.

phagocytic cells and the digestive tract of invertebrates and vertebrates. Between these two, in developmental terms, he cited the work of Ranvier on the resorption of dead nerve fibres, and Kowalesky's work on phagocytic leucocytes involved in the complex metamorphosis of fly larvae. Phagocytic-like activity is now recognised as a widespread biological phenomenon in tissue repair, but is marked in being a very much slower process than the classical neutrophil or macrophage-bacterial interaction. For example, chick embryonic retinal pigment epithelium takes about two hours to phagocytose latex particles, *in vitro*, although for some species these cells may exhibit a pre-uptake latent period of 12 hours (Tsunematsu *et al.*, 1981).

The vertebrate immune system appears to be considerably more sophisticated than that of invertebrates, and the phylogenetic origins have been much investigated in recent years (Wright, 1976; Warr *et al.*, 1977). Invertebrates rely principally on less specific defence mechanisms such as phagocytosis (Bang, 1973; Cheng, 1975; Rowley, 1981), even though "phagocytes" comprise only about 1% of the total circulating blood cell population (Endean, 1960). Furthermore, while there are variations between species, the naturally occurring haemagglutinins are not effective as opsonins (Rowley and Ratcliffe, 1980). Little is known of killing activity in invertebrate

phagocytes, but it appears that the oxygen-dependent pathways, for example involving myeloperoxidase, are poorly developed (Rowley, A.F., personal communication).

Metchnikoff's studies allowed him to theorise on the development of phagocytic systems, in part due to the incredible range of his experimental models. Beyond the observations in Daphnia, he studied anthrax, cholera, erysipelas, malaria, typhus and other infections in experimental animals (Metschnikoff, 1887). He showed, for example, that "microphages" were ineffectual in limiting anthrax infection in mice, guinea pigs and rabbits, but that the "macrophages of the spleen" appeared better able to cope. The ability of host cell populations to deal with anthrax bacilli was markedly enhanced, however, after a "weak vaccine" had been injected by the method of Pasteur, Chamberland and Roux. With erysipelas infection, Metchnikoff demonstrated the late arrival of macrophages in the inflammatory site, and how they effectively phagocytosed debris and effete microphages. He also demonstrated that bacteria may remain viable for protracted periods within phagocytic cells. Metchnikoff was fortunate in having enlightened patrons in this work, for example Prince Oldenbourg who took a keen interest in his typhus studies.

Metchnikoff and his colleagues were clearly prodigious workers, as pointed out by Binnie in the

preface to his English translation of Metchnikoff's book, "Immunity in Infective Diseases" (1905)*. The work includes chapters on plant immune mechanisms, touches on reproductive biology and gives a guide to regimes of immunisation.

While Metchnikoff's work was criticised by some contemporaries who saw humoral mechanisms of immunity as all-important, he himself fully realised the significance of serum factors, both per se and as a vital adjunct to phagocytic function. For example, in his book, among many others he cited the work of Bordet, Pfeiffer and, above all, Ehrlich. He made reference to the typhoid immunisation studies of Wright, who did much to consolidate the cellular/humoral basis of effective phagocytosis (Wright and Douglas, 1903).

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* Originally published in 1901, in French.

NEUTROPHIL HOMEOSTASIS

Metchnikoff's "microphages" are now referred to as granulocytes or polymorphonuclear leucocytes and are basophilic, eosinophilic or neutrophilic. Neutrophils constitute the largest circulating human leucocyte population, in a ratio of approximately 1:1000 with erythrocytes. The neutrophils are, of course, the phagocytes of primary cellular defence. They are the progeny of marrow stem cells, which mature to band and segmented forms over a period of about ten days before being released into the circulation (Klebanoff and Clark, 1978: 74-75). The marrow compartment is divided into the precursor pool and the mature granulocyte reserve, from which population functional cells pass into the vascular space (Fig. 1.1). In a normal healthy adult approximately 1×10^{11} granulocytes pass into the circulation per day and there the cells also exist as two "pools" in equilibrium. The circulating pool is that from which granulocytes are sampled by venepuncture; the more slowly moving marginating pool close to the vessel walls is the origin of cells which pass into the tissues, presumably under the influence of chemotactic factors. It is known that the granulocytes remain in the circulation for only a matter of hours, but how long they remain in the tissue compartment is not known (Klebanoff and Clark, 1978: 74-75). Indeed, the size

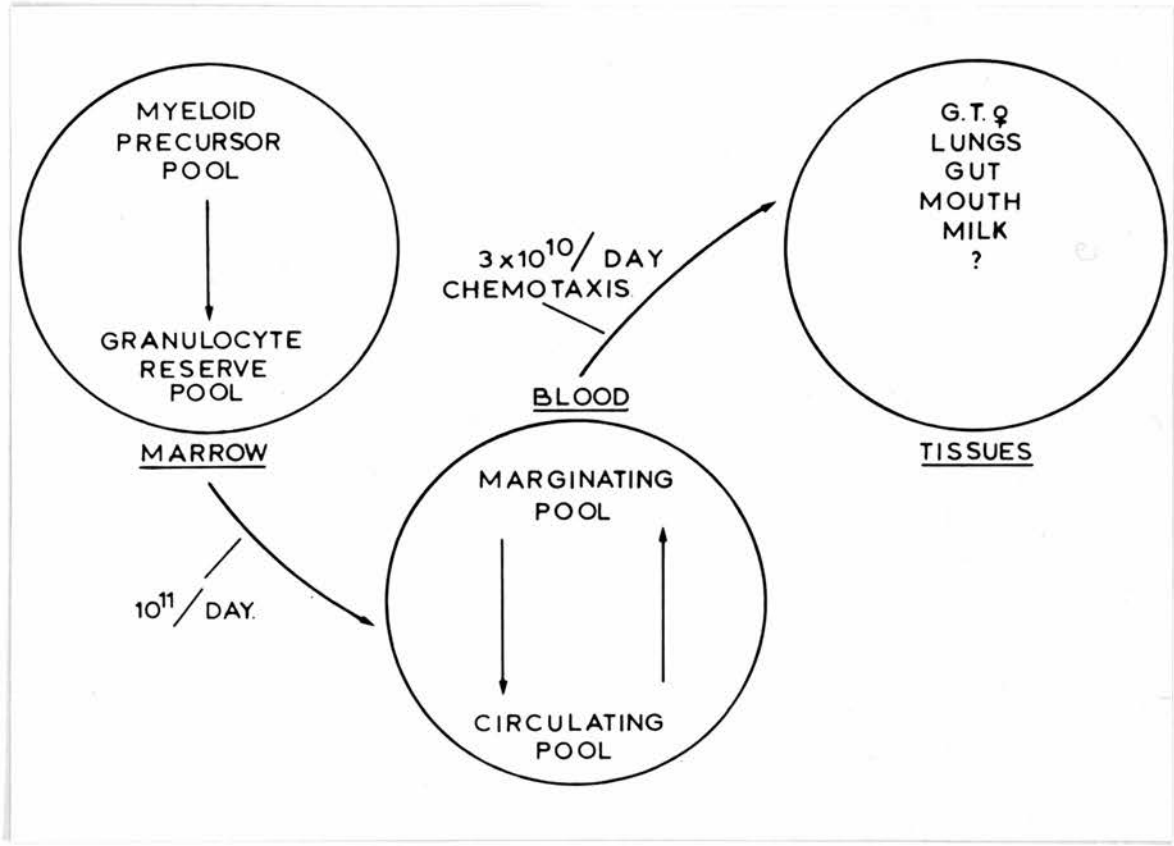

FIG. 1.1: Neutrophil homeostasis. Neutrophils are produced and mature in the bone marrow, from where they pass into the circulation and thence to the tissues. The relative proportions of cells migrating to different anatomical sites are unknown.

G.T.♀ = Genital tract, female.

and precise areas of migration are yet to be fully determined, and some examples of known tissue sites are illustrated in Fig. 1.1. While neutrophils and other leucocytes can be sampled from accessible secretions (e.g. colostrum) and exudates (e.g. gingival crevicular fluid), some workers have also attempted to estimate their proportions in connective tissue cells in an experimental system (Darcy and Dean, 1981).

The control of neutrophil production in the marrow is only now being elucidated. Detailed studies of cyclical neutropenia in Grey Collie dogs provided data regarding the time scale of cell production (Dale, Alling et al., 1972; Dale, Ward et al., 1972). In recent years interest has focused on so-called colony stimulating factors (CSF), a group of glycoproteins produced by the monocyte-macrophage system and possibly also by lymphocytes (Foster et al., 1968; Chervenick and Boggs, 1970; Golde et al., 1972; Baker and Galbraith, 1979; Gordon and Gordon-Smith, 1980; Hartmann et al., 1981). They induce DNA synthesis and cell proliferation at least in vitro and probably in vivo (Moore and Williams, 1973). Control of granulopoeisis is thus effected via a number of negative feedback loops. In addition, there is a recent report suggesting that bacterial clearance rates may also regulate and influence granulocyte production (Hartmann et al., 1981). Neutrophils themselves do not re-enter the circulation after passing into the tissues,

but this does not preclude the possible regulatory products of bacteria or even neutrophil-derived substances from the tissue site returning to the circulation.

Neutrophils comprise approximately 75% of the leucocytes sampled by venepuncture, the absolute count being on average higher in women than men, and higher in the afternoon than the morning (Bain and England, 1975). These authors also demonstrated a significant correlation between the peripheral blood neutrophil and monocyte counts, so supporting the hypothesis of a linked regulatory mechanism of production and release. However, as illustrated in Fig. 1.1, the circulating pool of granulocytes, while in equilibrium with the marginating pool, may not be identical to it at any given time point. The functional goal of neutrophils is to reach tissue sites in order to confront bacteria and it has been demonstrated experimentally that peripheral blood measurements of cell numbers do not accurately correlate with either the number of cells being produced or the number migrating to infected sites (Hartmann et al., 1981).

ANTIMICROBIAL FUNCTION OF NEUTROPHILS

The vital importance of an intact host defence system is epitomised in studies of the neutrophil. Defects of normal function are associated with recurrent, severe or protracted infections, while neutropenia is life-threatening, even in this antibiotic era. Over the past 25 years, although the neutrophil has received less attention by researchers than the lymphocyte, a voluminous literature on all aspects of neutrophil function has been produced. In the wake of these original reports have appeared reviews relating to those principal activities into which neutrophil physiology and pathology are by convention divided, i.e. chemotaxis (Wilkinson, 1979), phagocytosis (Stossel, 1974a,b,c), microbicidal activity (Klebanoff, 1975; Block et al., 1980; Root and Cohen, 1981; Segal, 1981) and functional abnormalities (Tauber, 1981). Investigations have reached a stage which allows the formulation of totally comprehensive reviews of the neutrophil literature (Murphy, 1976; Klebanoff and Clark, 1978).

Metschnikoff (1887) may have been the first to record the phenomenon of in vivo phagocytosis in man, in cells from the buccal cavity. He deduced that they had probably migrated from the pharyngeal tonsils and used this evidence in the development of his theory concerning the evolution of phagocytic defences in sites of primary

bacterial challenge.

Phagocytosis like chemotaxis depends on the integrity of microfilaments (e.g. actin and myosin) and the microtubular system as well as the complex interaction of electrolytes with these (Stossel, 1974; Klebanoff and Clark, 1978: 126-132). The phagocytic process operates effectively both in aerobic and anaerobic conditions although killing is generally impaired in the latter (McRipley and Sbarra, 1967a; Mandell, 1974; Ingham et al., 1981). However, it has become apparent that the many variables involved in phagocyte-microbial interaction, for example serum factors, pH, bacterial species and strain-specific surface characteristics, and oxygen tension, are of paramount importance in determining the outcome. Thus many experimental results are equivocal, and some anaerobes for example have been shown to profoundly influence their own phagocytosis and killing (Tofte et al., 1980).

Some recent studies have demonstrated that neutrophils have a considerable potential for extracellular killing of bacteria (Henson, 1980; Sips and Hamers, 1981), but the classical bactericidal events occur intracellularly, following phagocytosis (Klebanoff and Clark, 1978: Chs. 4 & 7).

The "respiratory burst" in neutrophils (De Chatelet, 1975; Klebanoff, 1975; Rossi et al., 1977) is the key

to subsequent microbicidal events, and may be initiated even before phagolysosomal formation is complete. This very complex and imperfectly understood area of neutrophil function centres around membrane-associated events (Elsbach, 1977; Henson, 1980). The energy requirement is obtained principally by anaerobic glycolysis, since neutrophils possess very few mitochondria (Elsbach, 1972; Klebanoff and Clark, 1978: 191-192). As a result of the respiratory burst, essential to which is the four-stage reduction of oxygen to water, a number of reactive molecules, ions and radicals are produced. For example, hydrogen peroxide, H_2O_2, (Klebanoff, 1975), superoxide anion (Babior et al., 1973; Johnston et al., 1975), the hydroxyl radical (Johnston et al., 1975), and singlet oxygen species (Krinsky, 1974; Allen, 1979) have all been shown to be bactericidal individually and may interact to potentiate killing (Allen, 1979). In addition, a powerful oxidising system is established if primary granule-associated myeloperoxidase interacts with halide and hydrogen peroxide (Klebanoff and Hamon, 1972; Sbarra et al., 1977). The consensus of opinion is that all these mechanisms are the basis of bacterial killing by neutrophils (Block et al., 1980), but recent work has suggested that the reactive oxygen species may be incidental to the sequence of reduction of oxygen to water. Reactive oxygen species also produce an environment for alternative killing mechanisms and modify

the intravacuolar pH (Segal, Geisow et al., 1981; Segal, 1983). It may be, of course, that all types of mechanism operate simultaneously to improve killing efficiency.

The bactericidal armamentarium of the neutrophil also includes the cytoplasmic granules and their contents. These were described in detail, including histochemistry and drawings made from microscopic observations, by contemporaries of Metchnikoff (Kanthack and Hardy, 1894). Once again there appears to have been a hiatus of over half a century before the work of Hirsch and Cohn regenerated interest in this area (Cohn and Hirsch, 1960; Hirsch and Cohn, 1960). Two populations of granules were described: azurophil (primary) and specific (secondary). In the mature neutrophil specific granules outnumber azurophil granules by approximately 2-3:1 (Klebanoff and Clark, 1978: 13-29), but species differences in granule proportions and constituents exist (Klebanoff and Clark, 1978: 29-35), which means that antibacterial mechanisms of neutrophils in animals and man are not always comparable. For example, only chicken neutrophils have lysozyme at levels similar to humans. Guinea pig neutrophils, often used in experimentation, contain approximately 10% as much myeloperoxidase as the human cell; chicken neutrophils have none (Rausch and Moore, 1975). Specific granules contain lactoferrin and about half of the cell's lysozyme

(Leffel and Spitznagel, 1972). Bactericidal activities of these agents are independent of oxygen. The azurophil granules contain the remainder of the lysozyme and the myeloperoxidase (Schultz et al., 1965). The lysosomes are a distinct population of granules containing the acid hydrolases (Segal et al., 1980). The neutral proteases and cationic proteins are also granule-associated (Spitznagel and Chi, 1963; Olsson and Venge, 1972; Elsbach, 1977; Klebanoff and Clark, 1978: 455-463).

A much less researched area of neutrophil function is the digestion and degradation of bacteria following killing. The rate at which some constituents are removed has been studied, particularly lipids (Elsbach, 1972). The role of polyelectrolytes in controlling degradation has recently received attention (Ginsburg et al., 1976; Ginsburg and Quie, 1980).

OTHER ACTIVITIES OF NEUTROPHILS

From clinical evidence alone, it is apparent that the neutrophil is of paramount importance, both in controlling pathogens and maintaining an equilibrium with respect to the commensal flora. This balance is delicate, and in the immunologically uncompromised individual, few clinical problems arise. However, there is increasing evidence that some forms of tissue damage may be mediated by, or caused by neutrophil activities.

It is important to maintain a perspective between host defence and destructive activities of neutrophils.

Extracellular lysosomal enzyme release is no longer considered to be the result of post-phagocytic cytolysis alone but as a factor contributing to the inflammatory process both destructively and beneficially. For example, it has been suggested that pulmonary injury may be caused by non-specific immunological enhancement of neutrophil phagocytic activity (Lanser and Saba, 1981), as well as by bronchoalveolar lavage fluids containing high levels of potentially destructive lysosomal hydrolases (Henson, 1980). Neutrophils contain collagenase (Lazarus et al., 1968) which may contribute to connective tissue breakdown, and in human periodontal disease this may be highly significant in conjunction with tissue damage caused by other lysosomal enzymes and

immunological mechanisms (Page and Schroeder, 1981). The role of extracellular enzyme release in tissue destruction has been reviewed (Murphy, 1976: 177-199; Henson, 1980). Much of this activity in the inflammatory site may be essential to the degradation and removal of endogenous and exogenous debris (Henson, 1980). Furthermore, myeloperoxidase-mediated killing, traditionally considered to operate intracellularly, has been shown to be extremely effective extracellularly (Odeberg et al., 1974; Sips and Hamers, 1981). Thus there is evidence for both destructive and protective sequelae to enzyme release, although the final outcome will also be determined by the extracellular buffering capacity (Henson, 1980), and the potential for the neutrophils themselves to inactivate existing enzymes (Voetman et al., 1981).

Finally, neutrophils may limit tumour growth (Clark and Klebanoff, 1975; Sendo et al., 1981), and contribute to such physiological processes as collagenolysis in the uterine cervix during parturition (Junqueira et al., 1980).

NEUTROPHIL DYSFUNCTION

Gross functional disorders of neutrophils are uncommon; that is, those which inevitably produce significant clinical problems. With increasing interest and sophistication in dissecting out the complex pathways of activity, the role of neutrophil dysfunction in the pathogenesis of common diseases will be elucidated (Segal, 1981).

The most dramatic clinical manifestations, illustrating the vital role of neutrophils in host defence, occur in patients with agranulocytosis, which may be acquired, e.g. following drug therapy (Harkness, 1981). Relative neutrophil depletion in the circulating pool or neutropenia exists in various forms, the most documented, apart from the effects of cytotoxic drugs, being the congenital cyclical neutropenias (Baehner, 1978), which result from a defect in stem cell proliferation. In such patients recurrent clinical infections, with advanced periodontal disease and oral ulcers, coincide with the neutropenic state. Other forms of neutropenia have been described, such as chronic benign neutropenia (Stahlie, 1956; Cutting and Lang, 1964), and syndromes of neutropenia with other immunological dysfunctions (e.g. Björkstén and Lundmark, 1976).

Defective neutrophil function has recently been comprehensively reviewed (Tauber, 1981). Lesions may be classified in terms of the established division of neutrophil functions (chemotaxis, phagocytosis, granule function, oxidative metabolism and killing), and examples in all these areas are documented. One of the most studied, but rare conditions, is that of chronic granulomatous disease (CGD) first described by Berendes et al. (1957) and which may result from an absence or inactivity of the membrane-associated cytochrome b essential for the respiratory burst (Segal and Jones, 1980). This prevents bacterial killing via the oxidative pathways, except in the case of some catalase negative bacteria which provide the neutrophils with hydrogen peroxide. The inability of CGD neutrophils to reduce nitroblue tetrazolium (NBT) after stimulation is a useful diagnostic test, and has been applied to prenatal diagnosis (Newburger et al., 1979).

Myeloperoxidase (MPO) is central to the oxidative killing mechanism of neutrophils (Klebanoff, 1975) and certainly each cell contains a large amount of this haem protein (Venge et al., 1978). With improved screening techniques, symptomless MPO-deficient patients have been identified; thus the precise role and importance of MPO is less certain (Parry et al., 1981). Clinical signs of deficiency may only appear when enzyme activity is less than about 10% of normal, partly because nature provides

more enzyme than is necessary, and partly because other compensatory mechanisms operate (Harkness, 1980).

The Chediak-Higashi syndrome occurs both in animals and man. It is associated with abnormal granule function, and increased susceptibility to infection (Wolff et al., 1972). Other biochemical defects in neutrophil function, such as glucose-6-phosphate dehydrogenase deficiency, exist and all these and the above conditions have been reviewed in considerable detail (Klebanoff and Clark, 1978).

There is a vast literature relating to acquired defects of neutrophil function, for example those caused by tobacco smoke (Corberand et al., 1980), alcohol (Brayton et al., 1970), fluoride (Gabler and Leong, 1979), antimicrobial drugs (Finch, 1980; Gnarpe and Belsheim, 1981), the contraceptive pill (Kvarstein and Gjønnaess, 1981) and other steroid drugs (MacGregor et al., 1974; Klempner and Gallin, 1978a). Neutrophil function may be depressed by bacterial infection (Hellum and Solberg, 1977; Repine et al., 1976). Direct inhibition of chemotaxis has been demonstrated in relation to particular pathogenic organisms (Shurin et al., 1979; Adamu and Sperry, 1981). Abnormalities are also associated with some primary disease states, for example diabetes mellitus (Mowat and Baum, 1971; Repine et al., 1980).

Functional problems arise in granulocytic leukaemia and other myeloproliferative states, as a result of the neoplasia, the antimitotic chemotherapy, infection or antimicrobial chemotherapy (Baehner et al., 1973; Odeberg et al., 1975; Humbert et al., 1976; Pinkerton and Robinson, 1976). Finally, it should be mentioned that not all drug effects are adverse; some may in fact enhance neutrophil function (Renz et al., 1974; Raeburn 1982; Grant et al., 1983).

CONCLUDING REMARKS

Metchnikoff and others at the end of the 19th century formulated theories of inflammation and immunity which have largely stood the test of time. Surprisingly, little progress was made regarding neutrophil function for about half a century, but in the past 25 years, there has been an enormous expansion of interest. Although considerable advances have been made, aided by electron microscopy and sophisticated biochemical and immunological techniques, much is still to be discovered especially in understanding the genetic basis and aetiology of functional neutrophil disorders. Recent evidence has suggested that a suppression of granulopoeisis by T-lymphocyte subsets may be responsible for some forms of neutropenia (Bogby, 1981). The biochemical lesion of chronic granulomatous disease has almost been elucidated and this condition may well be suited to genetic engineering should that become practicable (Segal, 1981).

The kinetics of neutrophil migration are now generally understood, but the fate of cells in the tissues is far less certain (Fig. 1.1). It is not clear whether or not the vast numbers of neutrophils which pass into the tissues daily are "wasted". A more teleological view, based on the evidence from severe deficiency states, is that the migration of functional cells holds the body's commensal population of bacteria in balance. Thus, in

neutropenia, congenital or acquired, and in chronic granulomatous disease, commensal organisms often cause clinical infections, for example in the mouth, lungs, gut and skin. Constant migration also establishes "pathways" for the subsequent release of even larger numbers of neutrophils if challenge by pathogens ensues. An appreciation of neutrophil physiology in tissue sites, exudates and secretions is thus central to achieving a complete understanding of their *in vivo* significance, and their role in the aetiology and pathogenesis of inflammatory conditions, such as chronic periodontitis. Most knowledge will be gained by comparison of cells sampled from more than one compartment. Neutrophils do not return to the circulation once in the tissues but in the case of bacterial infection, peripheral blood neutrophils will exhibit increased NBT reduction, despite the stimulus being peripheral (Park *et al.*, 1968). This implies that there must be some signalling system at the inflammatory site which has metabolic consequences (Harkness, 1981; Hartmann *et al.*, 1981).

AIMS OF THIS STUDY

The purpose of this study was to investigate some functional characteristics of gingival crevicular neutrophils both to increase understanding of their presumed protective role in the aetiology of human periodontal disease and as a model of cellular migration. The following investigations were proposed:

1) To collect crevicular neutrophils by a method suitable for cell quantitation, including differential counting, vitality testing, determining *in vivo* phagocytosis and performing basic histochemical tests.

2) To examine the integrity of the respiratory burst by nitroblue tetrazolium (NBT) reduction and compare this to activity in peripheral blood neutrophils.

The reducing activity of human saliva was also studied.

3) To investigate the presence and activity of myeloperoxidase (MPO) as an enzyme specifically related to microbicidal function. The influence of two antibiotics on MPO activity was studied in both crevicular and peripheral blood neutrophils.

4) To apply the above assays to a small group of patients who had diagnosed disorders of neutrophil production or function.

CHAPTER 2

GINGIVAL CREVICULAR NEUTROPHILS

INTRODUCTION

Leucocytes in the Oral Cavity

The principal function of the neutrophil as an essential antimicrobial system has been outlined in the previous chapter. For the healthy individual in whom no additional bacterial challenge exists, neutrophils marginate in small blood vessels and pass via the tissues onto mucosal surfaces. Thus, in secretions and exudates they limit the activity of commensal bacteria and contribute to the maintenance of host-microbial equilibrium.

The oral cavity is a site of such neutrophil-bacterial interaction, being extremely complex in that both anaerobic and aerobic habitats exist, with anatomical features as physically varied as, for example, the ventral surface of the tongue, periodontal pockets and the enamel tooth crowns. Finally, saliva and crevicular fluid, both extremely variable in constitution, further modify these interactions.

The presence of leucocytes in the oral cavity was noted by Metschnikoff (1887) who deduced that they originated from the tonsillar tissues. Some years later Mendel (1916), having demonstrated the presence of leucocytes, concluded that the gingival pocket may be

regarded as a 'lymphatic sac'. Lacquer (1911) showed, however, both morphologically and cytochemically that these cells were polymorphonuclear leucocytes. As with the study of functional characteristics of peripheral blood neutrophils, several decades separated these early observations from subsequent investigations on oral leucocytes. Hagerman and Arnim (1954) commented that epithelial cells and polymorphonuclear leucocytes were present in smears of gingival crevicular material from clinically healthy sites.

The gingival crevice is the principal route for entry of leucocytes into the oral cavity (Sharry and Krasse, 1960; Schiott and Löe, 1970) and the majority are neutrophilic polymorphonuclear leucocytes (Klinkhamer, 1963; Attström, 1970; Raeste, 1972a). The rate of entry of cells into the oral cavity as measured in saliva is approximately 1×10^5 cells per second in a fully dentate subject (Klinkhamer, 1963). Lower counts are obtainable from totally edentulous subjects with clinically healthy mucosa (Raeste, 1976). Initial studies of oral leucocytes were limited to salivary cells, collected by a mouth rinsing technique using buffered saline, which were shown to be 90% neutrophils and 10% unidentified mononuclear cells (Klinkhamer, 1963). Wright (1968), however, recorded only 58% neutrophils with very small percentages of other myeloid cells but 38% lymphocytes. More thorough methodology using a Millipore

filter technique for collection (Raeste and Calonius, 1971) showed that 98-99% of salivary leucocytes were neutrophils (Raeste, 1972a).

The first clinical application of salivary cell counting was to develop an objective measurement of gingival tissue inflammation. The rate of cell migration into the oral cavity, or the orogranulocyte migratory rate (OMR), was shown to correlate well with the inflammatory state. The oral migratory rate index (OMRI) was developed and accounted for the number of teeth present, a factor which would obviously govern the potential for cell migration (Klinkhamer, 1968a). Skougaard et al. (1969), and subsequently Woolweaver et al. (1972), demonstrated a positive correlation between OMR and the Gingival Index of Löe and Silness (1963). Other workers failed to confirm this either in children (Cox et al., 1974) or in adults (Hase and Reade, 1979).

Once neutrophils enter the saliva, their functional characteristics alter and over the past decade, with an increasing awareness of their role in the aetiology of periodontal disease, attention has been focused on more detailed aspects of their function. Cells in saliva, a hypotonic environment, tend to exhibit progressive degeneration (Sharry and Krasse, 1960; Raeste and Calonius, 1971; Raeste, 1972b) to an extent that may result in their being mistaken for mononuclear cells

(Raeste and Calonius, 1971). However, contact with saliva may increase some aspects of neutrophil function *in vitro* (Sela *et al.*, 1981; Ginsburg, personal communication) and such effects may be relevant to local host-bacterial interactions. The majority of studies are now conducted on leucocytes collected directly from the gingival crevice especially since a variety of simple, reliable sampling techniques have become available (Cimasoni *et al.*, 1977).

Leucocytes in Gingival Crevicular Fluid

Sueda *et al.* (1969) collected gingival crevicular fluid with filter paper strips. This did not permit quantitation and a more precise method was described by which crevicular material was removed with a sterile platinum loop (Lange and Schroeder, 1971). The two methods were then combined to examine eukaryote cells and bacteria in the gingival crevice (Lange *et al.*, 1972). Attström (1970) described a technique for examining cells which were adherent to Styroflex film and he reported a differential count of 97% neutrophils, with 2% monocytes and 1% lymphocytes. These results accorded with those of Raeste (1972a) for saliva, and also with those of Egelberg (1963) who collected gingival crevicular fluid with a capillary tube. These techniques permitted

quantitation as did that described by Hershon (1971), in which enzymes were assayed from crevicular fluid, collected in absorbent paper points. However, only small sample volumes were available when the gingivae were clinically healthy. A custom-built acrylic appliance was devised by Takamori (1963) and modified by Oppenheim (1970), with internal channels along the buccal and palatal margins to fit a dental arch precisely. Fluid was collected by means of a pump method, now used extensively in cellular and enzyme studies (Cimasoni, 1974; Kowashi et al., 1980). The disadvantage of this device is the technical expertise required and the inconvenience of having to construct one for each subject. The rinsing method of Skapski and Lehner (1976) employed a 50 µl microsyringe to introduce small volumes of Hank's balanced salt solution into the gingival crevice and then to re-aspirate the washings. The differential cell count was performed on Leishman stained samples and shown to be 91.5% neutrophils and 8.5% mononuclear cells. Viability of the fresh cell sample dispersed in a Neubauer chamber, by Trypan blue dye exclusion (McLimans et al., 1957; Fallon et al., 1962) increased from 81% for cells in the initial washings to 99% in later samples. The lower viability was ascribed to the early washings containing exhausted and degenerating leucocytes. This technique, sometimes in modified form, has been widely used for the more recent, detailed studies of crevicular

neutrophils, including one study in rats (Ramamurthy et al., 1979). Wilton et al. (1976) reported a differential count of 91% neutrophils and 8.8% mononuclear cells in man, which was almost identical to that of Skapski and Lehner (1976). They proceeded to show that the mononuclear population comprised 24% T lymphocytes, 58% B lymphocytes and 18% monocytes. Cell counts in human gingival washings were reported as approximately 1 x 10^5 neutrophils/ml; 85-87% were viable as tested with Trypan blue (Wilton et al., 1977a,b). Similar values were reported by Murray and Patters (1980), although they recorded cell numbers per site sampled. Scully (1982) found that 83% of crevicular neutrophils were viable and that approximately 20% showed evidence of in vivo phagocytosis. One of the difficulties with this washing technique is the lack of precise quantitation. For the original method devised by Skapski and Lehner (1976), Challacombe et al. (1978) calculated a dilution factor of between 1 in 20 and 1 in 50. Recovery of transfused neutrophils from gingival crevices in Rhesus monkeys showed that the concentration of cells was higher than that of peripheral venous blood, so supporting the view that there is a positive migration of cells, presumably under chemotactic stimulus (Scully and Challacombe, 1979). Finally, one study of crevicular neutrophils in beagle dogs utilised the acrylic appliance technique of Oppenheim (1970) combined with the aspiration method. The results

were similar to those in man, i.e. 86% of the cells were neutrophils, 8% mononuclear cells, with epithelial cells and debris comprising the remainder (Kraal and Bowles, 1977).

Gingival crevicular cells resemble peripheral blood neutrophils more closely than those from saliva when studied morphologically (Lange and Schroeder, 1971; Raeste, personal communication). This is perhaps not surprising since they have emerged into the crevicular fluid after a short passage from the gingival capillaries, through the connective tissue and junctional epithelium. The rate of flow of gingival crevicular fluid in dogs has been shown to vary directly with the clinical degree of inflammation, but this relationship is less precise for the migrating neutrophils (Attström and Egelberg, 1970; Attström, Graf-de-Beer et al., 1975). More recently, evidence has been presented to demonstrate that in humans also crevicular fluid and neutrophils enter the oral cavity under independent control (Kowashi et al., 1980). The leucocyte count was shown to vary with the inflammatory state of the gingival tissues (Attström, 1970; Scully and Challacombe, 1979) and to reflect systemic influences, e.g. as in diabetes (Ramamurthy et al., 1979).

In the classical concept of an inflammatory response, neutrophils migrate under the influence of chemotactic

stimuli. Clearly, the bacteria, their components and products in the crevice and saliva constitute an adequate and effective stimulus (Tempel et al., 1970; Lindhe and Helldén, 1972; Helldén et al., 1973; Kraal and Loesche, 1974; Lindhe and Socransky, 1979; Scully and Challacombe, 1979; Ginsburg and Quie, 1980).

Humoral Components of Crevicular Fluid

Complement components are also present in the gingival tissue (Brandtzaeg, 1973; Genco et al., 1974) and crevicular fluid (Shillitoe and Lehner, 1972). Activation occurs in the crevice (Attström, Laurel et al., 1975) and this may lead to subsequent tissue destruction via immunological responses (Allison et al., 1976). Equally it is likely to enhance neutrophil adherence via C5a (Gallin, 1980), chemotaxis via C5a des arg (Wilkinson and Lackie, 1979) and the binding of bacteria to neutrophils followed by phagocytosis and killing via C3b (Lay and Nussenzweig, 1968; Klebanoff and Clark, 1978). Crevicular fluid also contains immunoglobulins, principally IgG, which may further facilitate the antimicrobial activity of neutrophils in this site (Shillitoe and Lehner, 1972; Challacombe et al., 1978).

Gingival Crevicular Neutrophil Function

The first dynamic studies of crevicular neutrophils were undertaken to investigate the possible protective role of these cells in periodontal disease and dental caries. Electron microscopic studies in particular had shown that neutrophils in the crevicular region could phagocytose bacteria *in vivo* (Schroeder, 1970; Frank and Cimasoni, 1972; Garant, 1976) and the conclusion drawn from these observations was that they appeared to wall off plaque bacteria from the gingival tissue (Attström, 1975; Garant, 1976; Newman, 1980). It has already been mentioned that freshly stained preparations of crevicular cells show evidence of phagocytosis (Scully, 1982). *In vitro* studies in the same laboratory demonstrated that crevicular neutrophils from clinically healthy sites were less able to phagocytose *Candida albicans* blastospores than peripheral blood neutrophils, although killing was equally effective (Wilton *et al.*, 1977a). It was also shown that the decreased phagocytosis was probably due to impaired C3b receptor function (Wilton *et al.*, 1977b), possibly induced by the exudate itself. In patients with early or advanced periodontal disease, crevicular neutrophil viability and phagocytic capacity for latex particles were high (Murray and Patters, 1980). Phagocytosis, however, was markedly impaired in cells from patients with juvenile periodontitis, isolated from

diseased sites only. The latter finding was consistent with data from peripheral blood neutrophil studies in sufferers from this aggressive, early onset form of periodontitis (Cianciola et al., 1977; Clark et al., 1977; Lavine et al., 1979).

The bacteria usually associated with dental caries, both in animals and man, are the various serotypes of Streptococcus mutans (Fitzgerald and Keyes, 1960; Loesche et al., 1975). Serotype c is most frequently isolated in human caries (Duany et al., 1972) and in Rhesus monkeys fed a sucrose-rich diet (Lehner et al., 1975). It was hypothesised that crevicular neutrophils might form an important component of the defence mechanisms against these bacteria (Scully and Lehner, 1979a). Experimental evidence from subsequent in vitro studies supported this hypothesis both in Rhesus monkeys (Scully, 1980a) and man (Scully, 1982). The peripheral blood neutrophil phagocytic and bactericidal capacities exceeded those of crevicular cells, which were greater than those of salivary neutrophils. These experiments were conducted using streptococci opsonised with specific antisera. It had already been demonstrated that immunisation of monkeys with formalinised Streptococcus mutans, serotype c, induced a significant protection against caries (Lehner et al., 1975). One mechanism for such protection may be the induction of opsonising IgG antibodies, which facilitate phagocytosis and killing of

the streptococci both in the crevice and on the tooth surface (Lehner et al., 1976). Experiments using sera from serotype c immunised Rhesus monkeys showed that phagocytosis and killing were increased in comparison to that with non-immune serum, not only for that serotype, but for types e, a and d as well, presumably due to antigenic cross-reactivity (Scully and Lehner, 1979b).

The cumulative evidence suggests that crevicular neutrophils are functional, albeit in a reduced form as compared to those from peripheral venous blood. They appear to be more functional than salivary cells, although a recent study has demonstrated the reverse with respect to phagocytosis of Candida albicans and Salmonella typhi via complement receptors (Sela et al., 1981).

The limited number of studies of gingival crevicular neutrophils to date has therefore been in two groups, i.e. the specific and detailed work which relates to the protective immunological responses in dental caries and the investigations of neutrophil function which may bear relevance to the aetiology of periodontitis. With regard to the latter, neutrophils were mostly challenged with inert particles (latex) or Candida albicans, so that much work is required to determine how neutrophils respond to commensal bacteria which exist in periodontal pockets, particularly to those anaerobes implicated in destructive disease (van Palenstein Helderman, 1981). It has been suggested that variations in function of crevicular as

compared to peripheral blood cells may be a general feature of cells in an inflammatory exudate (Wilton et al., 1977a,b). Therefore it is relevant to investigate the gingival crevicular neutrophils further for possible relevance to the aetiology and pathogenesis of periodontal disease and also as a model of phagocytic cells migrating to primary sites of confrontation with commensal and pathogenic bacteria.

The first requirement in the present study was therefore to establish a suitable technique for collecting cells in gingival crevicular fluid, and subsequently to perform a series of basic investigations from which it would be possible to conduct more detailed functional studies.

The basic parameters to be measured were as follows:

- cell viability
- total cell counts
- differential leucocyte count
- an assessment of in vivo phagocytosis
- basic histochemistry, exemplified by neutrophil alkaline phosphatase (NAP) determination

MATERIALS AND METHODS

Collection of Cells in Crevicular Fluid

Initially, serial washings were attempted as described by Skapski and Lehner (1976) but using 1 ml or 2 ml disposable plastic syringes, with gauge 25 needles. Although some cells were collected in this way, the syringe and needle hub volumes were so large as to result in considerable loss of material. Subsequently, a 50 µl gas-tight Hamilton syringe (Hamilton Products, Bonaduz, Switzerland) was used as in the original method (Skapski and Lehner, 1976). Two needles were tested: a fixed needle/syringe unit (Hamilton No. 705-N) and a removable Hamilton, Luer-lock needle with appropriate fitting on the syringe (No. KF-7265). Both the fixed needle and removable needle (gauge 26, internal diameter 0.15 mm, with a 12^0 bevel) provided flexibility and the long, shallow bevel allowed penetration of the healthy gingival crevice without apparent trauma.

To summarise the method of collection, 10 µl aliquots of physiological rinsing fluid (PBS) were introduced into the gingival crevice being sampled by insinuating the bevelled needle tip along the dry tooth surface. By slowly withdrawing the plunger, diluted crevicular fluid containing cells was obtained. By

repeating the process several times and pooling washings from several sites, sufficient cells could be obtained for a number of investigations.

Cell Viability

Viability was assessed in leucocyte suspensions from interproximal sites in 10 subjects with no evidence of gingivitis according to the Gingival Index of inflammation (GI < 0.2; Löe and Silness, 1963). The investigation was repeated in a further five subjects with clinical gingivitis of varying degrees (GI = 1.0-3.0). The first two washings were discarded so as to remove the heavy contamination of epithelial cells, bacteria and possibly old leucocytes in the free crevicular fluid. It was thus hoped that subsequent washings would contain marginating cells. Subsequent aspirates were mixed on a clean microscope slide with 0.5% Trypan blue dye (British Drug Houses, Poole, England), a coverslip gently applied, and the preparation immediately examined at magnification x 312.5 with a Zeiss RA microscope (Carl Zeiss (Oberkochen) Ltd., London). Viability was determined as a percentage by scoring the number of leucocytes excluding the dye per 100 cells counted 10 times per subject. This method readily permits distinction between vital (clear) and non-vital (blue) cells. A total of 1,000 cells per subject were thus counted.

The Cytocentrifuge

This was the single most important item of equipment for the basic investigations, including histochemistry. The cytocentrifuge (Shandon Southern Products Ltd., Cheshire) allows relatively small numbers of cells in small volumes of fluid to be concentrated into a monolayer within a 6 mm diameter circle on a standard glass microscope slide. The bulk of the suspending fluid passes out radially into an absorbent filter paper strip placed between the glass slide and the centrifuge well (Fig. 2.1). The manufacturers recommend that the minimum volume used is 0.1 ml. Any less causes the cell collecting area to be incompletely filled. This was borne out by experience. For leucocyte preparations the manufacturers suggest centrifugation speeds of approximately 1500 rpm for 5-10 minutes. However, a series of preparations was made of gingival cells collected from one donor (29 years of age, male), centrifuged for various speeds and for varying times in order to determine the optimum cycle to maintain morphological quality for examination. Preparations were stained routinely with Leishman stain. Morphological integrity was scored subjectively as follows:

0 = complete disruption of cells

1 = cells generally distorted

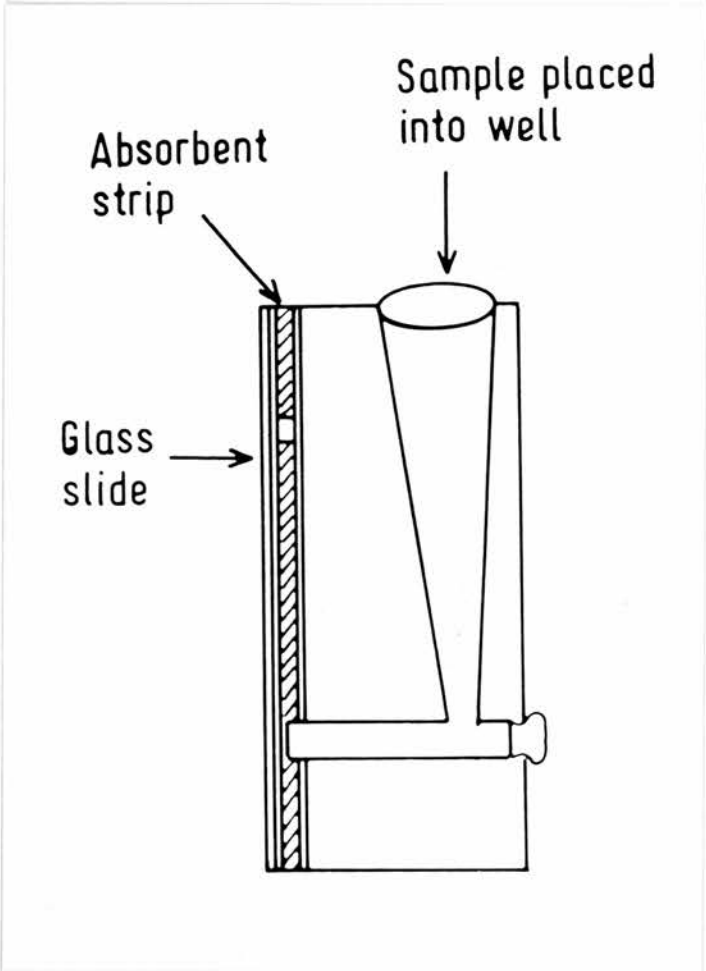

FIG. 2.1: The cytocentrifuge well assembly. Twelve of these are accommodated within the centrifuge head.

2 = < 50% of cells well preserved, the majority distorted

3 = 50-75% of cells well preserved

4 = overall maintenance of cell morphology

Effect of Storage

Once a suitable cytocentrifugation cycle had been determined, the possibility of storing the cells was investigated. Cells were collected and maintained in the plastic tubes at 37°C in a water bath, at 4°C in a refrigerator, or standing on the laboratory bench at room temperature (approximately 20-22°C). These were maintained for varying time periods before spinning onto glass and morphological assessment was made as before.

Cell Number, Differential Leucocyte Count and *in vivo* Phagocytosis

To determine these three parameters, cytocentrifuge preparations of crevicular washings were made as follows. Fifty microlitres of rinsing fluid were placed into a 2 ml cylindrical plastic tube (Searle, High Wycombe, Bucks.), and 20 serial samples were added. From this final dilution, 0.1 ml was placed in the base of a cytocentrifuge

well and the glass slide preparation made by centrifugation at 400 rpm for 5 minutes. The slides were stained immediately with Leishman stain.

Cell number

The cytocentrifuge is not recommended for quantitation. The manufacturers quote the results of comparing microscopic field counts of sheep erythrocytes to spectrophotometric density, from centrifugation volumes of 0.2 ml. To determine whether a reasonably accurate count was possible, the following method was employed. The 0.1 ml cell sample was spun onto a glass slide and stained as described above. The preparation was examined at x 500 magnification with oil immersion. By traversing the total 6 mm diameter several times it was possible to determine that this diameter constituted 14 complete fields. The edge of the preparation was not always precisely demarcated. The following area calculation was performed:

a) Diameter of one field of view = 1 unit

b) Diameter of total preparation = 14 units

Πr^2 = area of circle

$\therefore$ Area of one field of view = $\Pi \times 0.5^2$ =

$3.141 \times 0.25 = 0.7853$ units

Area of total preparation = $\Pi \times 7^2$ =

$3.141 \times 49 = 153.94$

$\therefore$ Ratio of total area to one field of view area

$= \frac{153.91}{0.7853} = 195.988$

Thus approximately 196 field areas constituted the total.

Assuming an even distribution of cells within the total area of the preparation made from 0.1 ml of fluid, a calculation for the cell concentration was performed:

cell count/field x 196 x 10 = total number of cells/ml

This was tested for preparations from ten healthy adult subjects with differing states of gingival health. Ten random field counts were made per subject in order to establish a mean value; the standard error of the mean was calculated from the standard deviation.

Simultaneously, counts were performed using a standard laboratory Neubauer counting chamber (Gallenkamp, East Kilbride, Glasgow) for five of the subjects.

An additional set of results was calculated from washings taken from two healthy young children to determine any obvious differences between these and the adults. These results were not included in the overall analysis.

Differential leucocyte count

From the same cytocentrifuge preparation it was possible to perform a differential leucocyte count. Five random counts, each of 100 leucocytes per slide, were made. Two preparations were made for each subject so allowing a mean count of 1,000 cells to be obtained.

Differentiation was based on standard nuclear and cytoplasmic morphology and staining, according to a standard text (McDonald, Dodds and Cruickshank, 1970). The clinical state of the tissues from which the cells were obtained was noted. Care was required because there was a tendency for nucleated epithelial cells with contracted cytoplasm to resemble large lymphocytes. Neutrophil granulocytes with severe degeneration exhibited rounded nuclei and were thus subject to confusion with mononuclear cells. This phenomenon has already been described for neutrophils in saliva (Raeste and Calonius, 1971). On careful examination, however, the nuclear staining between cell types was quite different.

Further differential counting was performed on cytocentrifuge preparations stained by the combined method for non-specific and chloroacetate esterases (Appendix 1). This method is used routinely in the Haematology Department of the Western General Hospital, Edinburgh and is based on individual reactions of neutrophils and monocytes. With α-naphthyl butyrate as substrate, the reaction is strong in monocytes and weak in neutrophils but, using naphthol AS-D chloroacetate, the reverse occurs. Lymphocytes are non-reactive. The reaction principle is based on the hydrolysis of a synthetic substrate (an ester derivative of naphthalene) by the leucocyte esterases. A naphthyl compound is

liberated which reacts rapidly with a diazonium salt to produce a coloured precipitate in the cell cytoplasm (Moloney, McPherson and Fliegelman, 1960; Li, Lam and Yam, 1973; Cline, 1981). The method is considerably more complex than that for Leishman stain, so that a series of counts using each method was performed on five subjects and compared.

In vivo phagocytosis

It was found that standard Romanowsky staining (Leishman stain) permitted an assessment of in vivo phagocytosis in crevicular exudate neutrophils. Controversy exists regarding the accuracy of microscopic evaluation of phagocytosis since it is possible to mistake extracellular, adherent bacteria for intracellular organisms. However, the method is widely used and, with care and experience, can be reliable (Klebanoff and Clark, 1978: 163-166). In this study only one observer was involved so that errors in judgement would be consistent. Bacteria were recorded as intracellular only if the outline of a phagocytic vacuole was visible. Mean values were determined from 10 fields.

The Relative Maturity of Crevicular Neutrophils

An additional investigation was conducted into the state of neutrophils in the gingival crevice to determine if the cells in the first washings were in fact degenerating forms. It is possible to establish the relative maturity of cells from their nuclear morphology using the Arneth-Cooke count as described by Britton (1969). Five stages for peripheral blood neutrophils are described, from juvenile to fully segmented forms, based on the extent of nuclear segmentation.

From five healthy adult subjects a cytocentrifuge preparation was made of the pooled first washings of five crevices. Three subsequent washings from the same crevices were discarded, and the fifth washings were again pooled, centrifuged onto glass and stained. The Arneth-Cooke counts were performed at x 500 magnification and compared. An additional category was included to allow for recording of degenerating cells which are not normally seen in peripheral blood films.

Neutrophil Alkaline Phosphatase (NAP)

The histochemical test reaction for NAP depends on a diazo-dye coupling. That is, the substrate (naphthol AS-MX phosphate) is hydrolysed by the alkaline phosphatase

to aryl naphtholamide and phosphate. The aryl naphtholamide is coupled to a diazonium salt to form an insoluble precipitate which can be seen microscopically.

The test method employed was that of the Haematology Laboratory, Western General Hospital, Edinburgh, which is based on the method of Kaplow (1963) (see Appendix 2). Freshly prepared films from peripheral venous blood and crevicular washings from six healthy adult subjects with no clinical evidence of gingivitis were prepared in order to determine whether crevicular neutrophils exhibited NAP, and if so how this related to peripheral blood NAP. The results were expressed as a percentage of cells staining positively for NAP and a score ascribed according to intensity, as described by Cline (1981); see Table 2.1. Thus, for 100 cells, all positive, a maximum score of 400 was possible.

TABLE 2.1:

CRITERIA FOR NAP SCORING

Score per cell	Percentage of cytoplasmic staining	Intensity of staining
0	0	0
1	< 50	Faint
2	50-80	Moderate
3	80-100	Strong
4	100	Intense

Adapted from Cline, M.J., 1981: 135.

RESULTS

Cell Viability

The viability of crevicular neutrophils for the ten subjects with no clinical evidence of gingivitis, mean age 28.7 years, and the five with gingivitis, mean age 26.6 years, is shown in Table 2.2. The mean percentage cell viability was 75.8 ± sem 1.2 and 74.8 ± sem 0.6 respectively.* There was no sex difference.

Figure 2.2 shows the progressive degenerative change in one glass-adherent cell. This was photographed while being observed during one of the viability assessments. It can be seen that the cell became enlarged and distorted as the blue dye entered the cytoplasm and nucleus.

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* The standard error of a percentage is calculated from the formula

$$SE = \sqrt{\frac{pq}{n}}$$

p = percentage vital cells
q = 100-p
n = total number of counts

However, this can only be applied to a single value and not to each of ten constituent counts from which a final mean was obtained for each subject in Table 2.2. Neither would there have been an indication of any possible variation between the ten counts of 100 cells. To allow for this, errors of percentage figures are quoted as standard errors of the mean (sem) throughout. This gives larger error values than the SE %.

As an example, the SE % calculated on the mean viability of 78.7% for the first subject in Table 2.2 was 1.29, in contrast to 1.76 for the sem (p = 78.7, q = 21.3, n = 1000).

TABLE 2.2:

VIABILITY OF GINGIVAL CREVICULAR NEUTROPHILS ASSESSED BY TRYPAN BLUE DYE EXCLUSION

	Sex	Age (yrs)	Viability/100 cells 1	2	3	4	5	6	7	8	9	10	Mean %	sem
	F	24	74	77	88	89	77	76	76	77	73	80	78.7	1.8
NO	F	32	78	84	86	83	71	86	79	80	75	75	79.7	1.6
	F	21	69	86	76	75	77	76	69	81	61	80	75.0	2.3
CLINICAL	F	34	78	54	76	81	73	76	78	80	91	59	74.6	3.4
	F	28	77	82	69	68	72	80	84	64	74	78	74.8	2.1
GINGIVITIS	M	29	89	78	84	88	78	80	71	72	81	76	79.7	1.9
	M	28	74	72	81	52	74	74	94	62	77	75	73.5	3.5
GI < 0.2	M	29	76	75	86	76	75	69	76	81	88	68	77.0	2.0
	M	25	65	69	75	68	76	53	77	59	75	60	67.7	2.6
	M	37	69	69	62	81	87	88	84	75	84	78	77.7	2.8
	Group	28.7 ± sem 1.6											75.8	1.2
CHRONIC	F	22	78	80	76	78	72	69	67	91	80	68	75.9	3.2
	F	29	82	71	62	74	83	71	87	75	76	77	75.8	3.2
GINGIVITIS	M	28	61	80	79	72	73	62	72	90	81	88	75.8	4.3
	M	24	71	56	90	83	67	71	74	79	81	58	73.0	4.8
GI = 1.0-3.0	M	30	76	86	84	57	65	75	83	67	68	76	73.7	4.2
	Group	26.6 ± sem 1.08											74.8	0.6

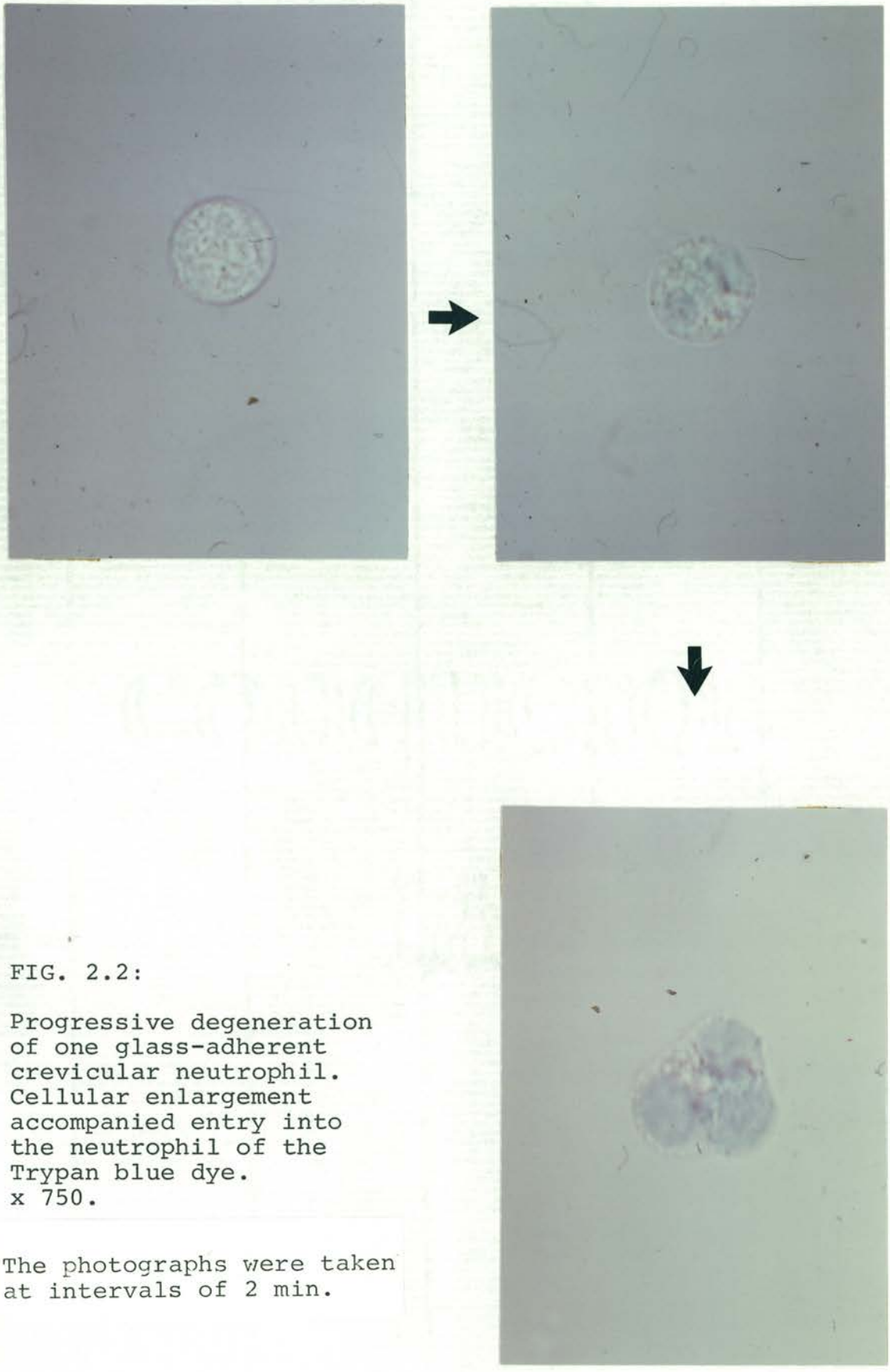


FIG. 2.2:

Progressive degeneration of one glass-adherent crevicular neutrophil. Cellular enlargement accompanied entry into the neutrophil of the Trypan blue dye.
x 750.

The photographs were taken at intervals of 2 min.

Operation of the Cytocentrifuge

The subjective assessment of the morphological integrity of neutrophils following various centrifugation cycles is shown in Table 2.3. Very slow speeds produced poor quality cells and low yields, particularly when the time was short. On the other hand, the speed recommended by the manufacturers (1500 rpm) produced equally poor results for these exudate cells. The best morphological appearances were produced by a cycle of 400 rpm for 5 minutes and almost as good by 500 rpm for 5 minutes. The slightly slower speed, however, subsequently proved to be more consistently reliable.

In order to determine if a longer centrifugation time would improve the yield, speeds of 200 and 300 rpm were used for up to 15 minutes in a subsequent experiment. The cell yield improved but morphological appearances did not. All subsequent preparations were therefore made by a centrifugation cycle of 400 rpm for 5 minutes. At this rotation speed, the *g* force was calculated to be 14.3 according to the standard formula:

$g = 1118 \times 10^{-8} \times r \times rpm^2$, where r = radius of extended centrifuge head in cm.

$\therefore \quad g = 1118 \times 10^{-8} \times 8 \times 400^2 = 14.3$

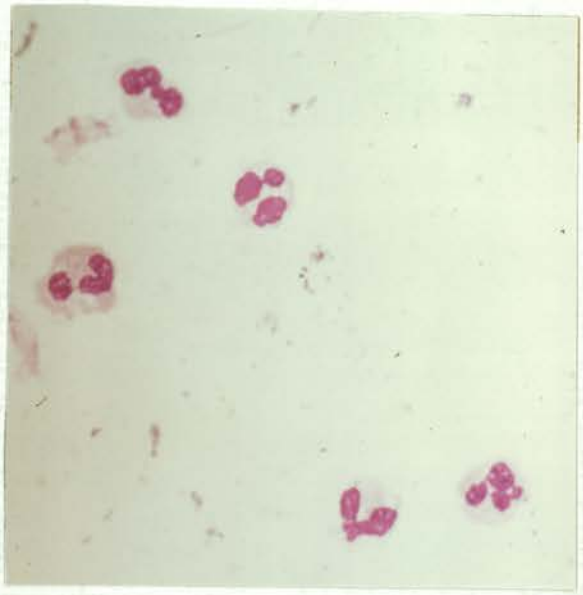
Figures 2.3a and b show examples of neutrophil preparations produced under these conditions, and Fig. 2.3c

TABLE 2.3:

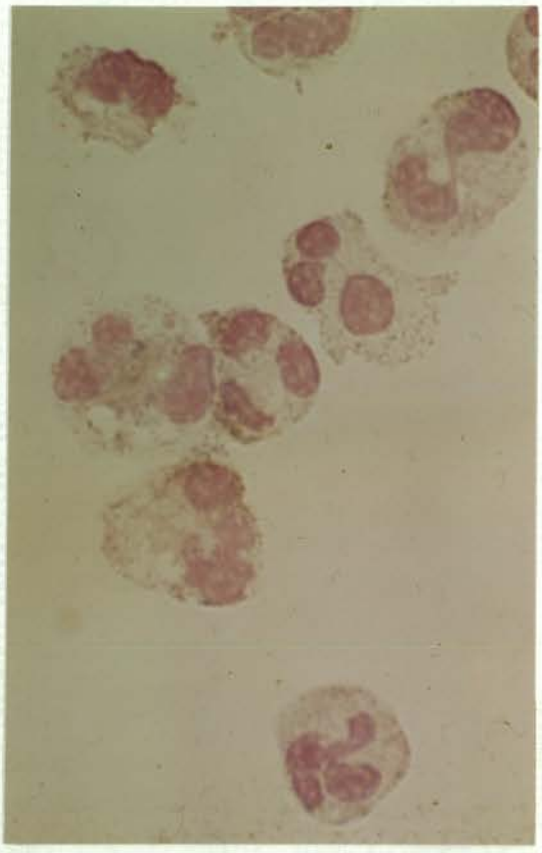
RESULTS OF INVESTIGATION TO DETERMINE THE OPTIMUM CENTRIFUGATION CONDITIONS FOR GINGIVAL CREVICULAR CELLS

Centrifugation Speed (rpm)	Time of Cycle (min)	Quality of Preparation
200	1	0*
200	2	0*
200	3	1*
200	5	1*
300	2	0*
300	4	1*
300	5	2
400	2	2
400	5	4
400	10	3
500	2	1
500	5	3
500	10	3
1000	1	1*
1000	5	2
1500	1	1*
1500	5	1*

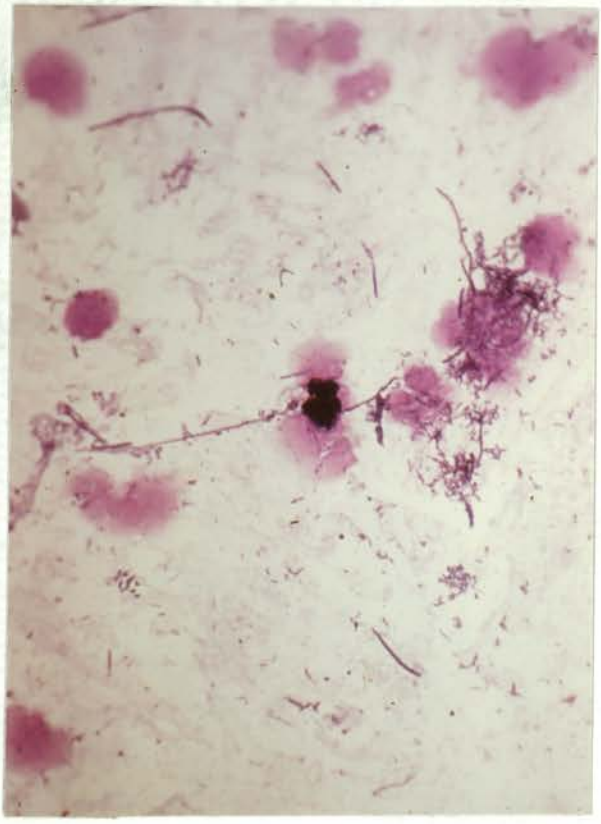
* Cell yields very low.



a



b



c

FIG. 2.3:

Cytocentrifuge preparations of gingival crevicular neutrophils.

a) Morphology well preserved. Leishman stain. x 480

b) Morphology well preserved. Wright's stain. x 1200

c) Cells totally disrupted. Leishman stain. x 480

Centrifugation cycle for a) and b) = 400 rpm for 5 min; for c) = 200 rpm for 5 min.

an example in which considerable cellular disruption has occurred.

Effect of Storage

The effect of storing the cells in the collecting tubes at three temperatures over varying time periods is shown in Table 2.4. Clearly the preservation of cellular morphology was not affected by storage or the temperature of storage up to 30 minutes. Beyond that time, progressive degeneration occurred at all temperatures, this being most marked at $4^{0}C$. Based on these findings in subsequent work every endeavour was made to minimise the interval between collection of cells and experimentation.

Cell Number, Differential Leucocyte Count and *in vivo* Phagocytosis

Crevicular cell numbers, differential leucocyte count and *in vivo* phagocytosis were all determined from the same cytocentrifuge preparations.

TABLE 2.4:

EFFECT OF STORAGE OF CELLS AT 37^0C, 4^0C AND ROOM TEMPERATURE (RT = 20-22^0C), PRIOR TO CENTRIFUGATION AT 400 RPM FOR 5 MINUTES

Time of Storage (min)	Quality of Preparation		
	37^0C	4^0C	RT
10	4	4	4
30	4	4	4
60	3	2	3
120	2	1	3
360	2	1	1

TABLE 2.5:

CREVICULAR NEUTROPHIL COUNTS FROM ONE FEMALE SUBJECT, AGED 34 YEARS

Cell count/field	Cell count/ml x 196 x 10
18	35,280
19	37,240
10	19,600
16	31,360
20	39,200
23	45,080
16	31,360
15	29,400
17	33,320
17	33,320
Mean ± sem	33,516 ± 2,131 neutrophils/ml washings

Cell Number

Table 2.5 shows a worked example for one subject of the calculation for mean neutrophil numbers/ml washings. Table 2.6 shows a comparison for the mean counts of five subjects between this microscopic method and that using the traditional counting chamber. It is apparent that the former procedure gave neither consistently higher nor lower values than the traditional method, and the values for each method were similar in magnitude. Statistical comparison of the mean figure for the microscopic method with the single figure obtained from the traditional method was as follows:

$$\frac{\text{Value}_1 - \text{Value}_2}{\text{sem for micro method}} = t$$

For 9 degrees of freedom, the significance level was obtained from tables. The results are shown in Table 2.6. In only one case was a statistically significant difference noted.

The mean values for the counts as performed by the microscopic method for the ten subjects with differing clinical gingivitis are shown in the composite Table 2.7. Clearly the values were extremely variable with, in most cases, large standard errors of the mean. No relationship was apparent between sex or age and count, or indeed the Gingival Index.

TABLE 2.6:

CREVICULAR NEUTROPHIL COUNTS FOR FIVE SUBJECTS DETERMINED BY THE "MICROSCOPIC" METHOD AND A HAEMOCYTOMETER/COUNTING CHAMBER

Subject		Neutrophil count/ml			
Age (yrs)	Sex	Microscopic ± sem	Neubauer Chamber	t value	Significance p
34	F	33,516 ± 2,131	30,000	1.6499	NS
17	F	96,122 ± 2,512	90,000	2.44	< 0.05
23	F	56,448 ± 6,211	65,000	1.3769	NS
29	M	44,820 ± 6,084	45,000	0.0295	NS
24	M	135,240 ± 19,825	120,000	0.7687	NS

TABLE 2.7:

CREVICULAR NEUTROPHIL COUNTS, DIFFERENTIAL LEUCOCYTE COUNT AND *IN VIVO* PHAGOCYTOSIS DETERMINED FROM LEISHMAN STAINED PREPARATIONS FROM TEN HEALTHY ADULTS AND TWO CHILDREN

Sex	Age (yrs)	GI	Neutrophil count/ml Mean ± sem	Differential count (%) ± sem Neutrophils	Monocytes/ Macrophages	Lymphocytes	% cells showing *in vivo* phagocytosis Mean ± sem
F	34	0.2	33,516 ± 2,131	89.9 ± 0.8	6.2 ± 0.6	3.9 ± 0.7	24.5 ± 1.8
F	23	0	56,448 ± 6,211	98.5 ± 0.2	1.5 ± 0.2	- ± -	17.8 ± 2.3
F	17	1.0	96,122 ± 2,512	98.1 ± 0.4	1.9 ± 0.1	- ± -	31.6 ± 2.0
F	28	0	62,490 ± 1,984	100 ± -	- ± -	- ± -	21.1 ± 0.8
F	22	1.5	41,564 ± 2,639	99.1 ± 0.1	0.9 ± 0.4	- ± -	36.2 ± 2.6
M	30	0.5	169,540 ± 3,706	96.8 ± 0.2	2.2 ± 0.2	1.0 ± 0.5	33.1 ± 0.6
M	24	1.5	135,240 ± 19,825	95.6 ± 0.5	2.3 ± 0.6	2.1 ± 0.5	20.8 ± 1.3
M	29	0.2	44,820 ± 6,084	90.5 ± 0.4	5.5 ± 0.5	4.0 ± 0.6	26.6 ± 1.8
M	46	0.1	67,248 ± 2,362	97.0 ± 0.3	3.0 ± 0.3	- ± -	12.0 ± 1.6
M	37	0	56,602 ± 3,543	97.2 ± 0.5	2.6 ± 0.3	0.2 ± 0.1	19.2 ± 1.2
Group	29.0 ± sem 2.7		76,359 ± 14,011	96.3 ± 1.1	2.6 ± 0.6	1.1 ± 0.5	24.3 ± 2.4
M	2.5	0	16,200 ± 1,950	97.0 ± 0.5	2.9 ± 0.5	0.1 ± 0.1	5.5 ± 1.2
M	3	0.5	1,764 ± 621	100 ± -	- ± -	- ± -	72.2 ± 14.0

The two small healthy boys had low cell counts, one particularly so. The differential count was similar to that for the adults, with a very high score for in vivo phagocytosis for one (Table 2.7).

Differential leucocyte count

The differential white cell counts as determined by the cytocentrifuge preparations of the same ten subjects are shown in Table 2.7. The vast majority of leucocytes in all cases were neutrophils with a very small proportion of mononuclear cells. In one adult, and one of the small children, the neutrophils constituted 100% of the leucocytic population. It is important to note that the standard errors were small for each subject and the overall group means, which was not the case for the total cell counts. There was no correlation between the differential count and either the inflammatory state of the gingival tissues or the total cell count for each individual.

For the five subjects in whom the differential count was repeated on a separate occasion and compared with that using the esterase staining characteristics, the results are presented in Table 2.8. In all cases, the proportion of neutrophils identified by the more specific staining method was higher, suggesting that with the Leishman stained preparations, in which reliance was

TABLE 2.8:

COMPARISON OF TWO STAINING METHODS IN DETERMINATION OF DIFFERENTIAL LEUCOCYTE COUNT

FROM GINGIVAL CREVICULAR WASHINGS IN FIVE SUBJECTS

Differential Count - Leishman Stain % mean ± SD			Subject No.	Differential Count - Esterase Staining % mean ± SD		
Neutrophils	Monocytes/ Macrophages	Lymphocytes		Neutrophils	Monocytes/ Macrophages	Lymphocytes
92.6 ± 1.8	4.9 ± 2.2	2.5 ± 2.1	1	98.8 ± 0.9	1.2 ± 0.8	-
92.8 ± 1.4	5.1 ± 3.1	2.1 ± 1.6	2	99.0 ± 1.5	1.0 ± 1.1	-
89.6 ± 0.9	9.3 ± 3.1	1.1 ± 1.4	3	97.8 ± 1.6	1.5 ± 1.4	0.7 ± 1.0
95.4 ± 1.9	4.6 ± 1.8	-	4	100	-	-
97.3 ± 1.8	2.3 ± 1.2	0.4 ± 0.9	5	99.1 ± 0.8	0.8 ± 0.9	0.1 ± 0.3
Mean ± SD 93.5 ± 2.9	5.3 ± 2.5	1.2 ± 1.1		98.9 ± 0.8	0.9 ± 0.6	0.2 ± 0.3
sem 1.3	1.1	0.5		0.4	0.3	0.1

placed on morphological and granular characteristics, some neutrophils were scored as mononuclear cells. Figure 2.4 shows the clear distinction between granulocytes and a monocyte in a preparation stained for esterase activity.

In vivo phagocytosis

Figure 2.5a illustrates the extracellular adherence of bacteria to the surface of a neutrophil, while Fig. 2.5b shows bacteria within a phagocytic vacuole. Only examples of the latter were scored as evidence of phagocytosis in vivo. The values are also given in Table 2.7 and demonstrate a wide variation within and between individuals. The results demonstrate that neutrophils within the crevicular exudate have considerable phagocytic capacity.

The Relative Maturity of Crevicular Neutrophils

For the five subjects studied, the results of Arneth-Cooke counts are shown in Table 2.9, as means for the group. For the 100 cells counted according to the method, it is interesting that, apart from approximately 5% degenerating forms in the first washings collected, the count was very close to that given for a peripheral blood film (Britton, 1969). In the fifth washing, the small number of degenerating forms was still present, but

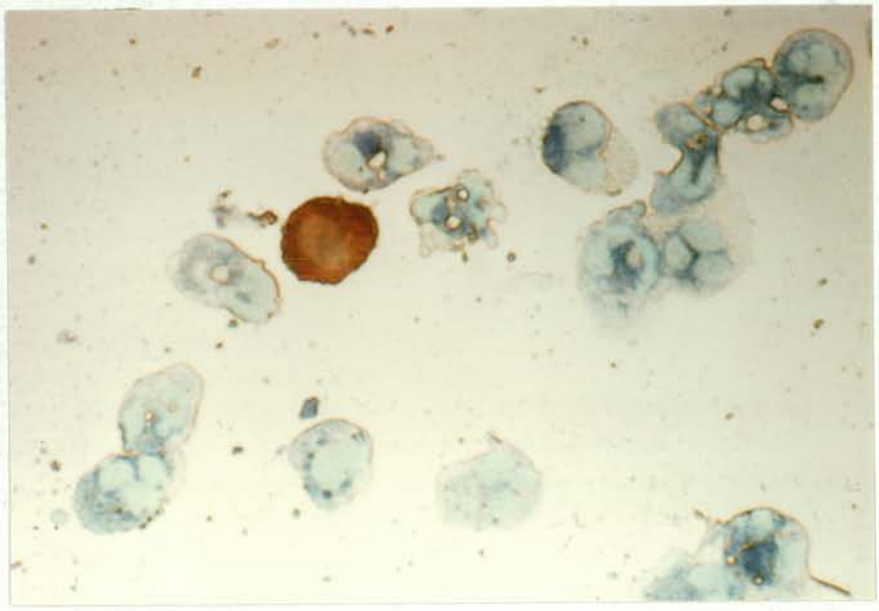


FIG. 2.4: Cytocentrifuge preparation of crevicular neutrophils stained for esterase activity. The green/blue stained granulocytes are clearly distinguished from the single macrophage.
x 750

FIG. 2.5a: Bacterial adherence to the surface of a crevicular neutrophil.
Leishman stain. x 1890.

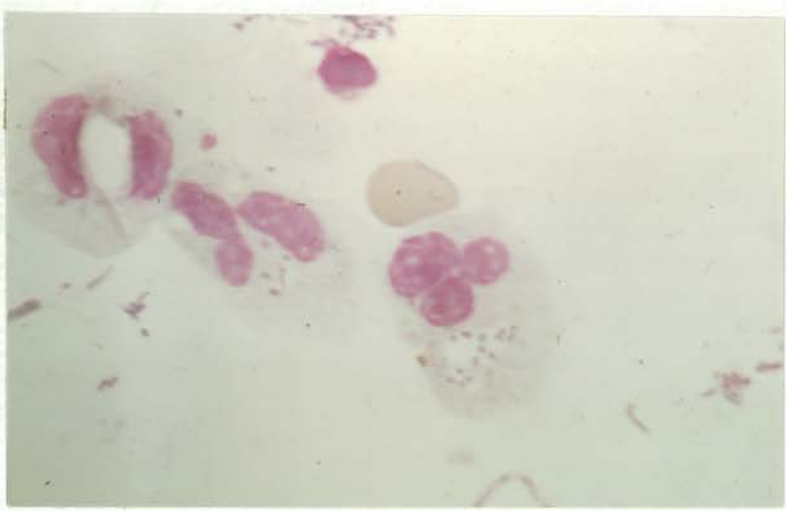


FIG. 2.5b: Intracellular bacteria in crevicular neutrophils, demonstrating <u>in vivo</u> phagocytosis.
Leishman stain. x 1890.

TABLE 2.9:

MATURITY OF GINGIVAL CREVICULAR NEUTROPHILS

Arneth-Cooke Counts from Five Adult Subjects
(mean % cells in each category ± sem)

Washing No.	I	II	III	IV	V	Degenerate cells
1	13 ± 4	23 ± 6	45 ± 6	13 ± 5	1 ± 2	5 ± 3
5	25 ± 7	32 ± 5	23 ± 5	15 ± 6	1 ± 2	4 ± 4
Normal peripheral blood neutrophils*	10	25	47	16	2	-

*Figures taken from Britton, C.J.C. (1969)

TABLE 2.10:

PERCENTAGE OF POSITIVE CELLS STAINING FOR NAP AND TOTAL SCORES FOR PERIPHERAL BLOOD AND GINGIVAL CREVICULAR NEUTROPHILS, IN SIX ADULT SUBJECTS

		Blood		Crevice	
Sex	Age (yrs)	% +ve cells	Score	% +ve cells	Score
F	34	69	133	75	113
F	28	51	78	87	168
M	46	64	94	76	132
M	30	58	91	82	156
M	29	67	138	79	143
M	40	68	142	95	187
	Mean	63	113	82	150

a considerable 'left shift' had occurred, indicating that less mature forms were entering the crevice.

Neutrophil Alkaline Phosphatase (NAP)

Both peripheral blood and gingival crevicular neutrophils stained positively for NAP. An example of a positively staining neutrophil in a peripheral venous blood film is shown in Fig. 2.6a, to which was ascribed a score of 2. Figure 2.6b shows a preparation of crevicular neutrophils exhibiting a range of positive staining. For the six healthy subjects studied, the percentage positive cells and scores ascribed in each neutrophil population are shown in Table 2.10. A higher proportion of crevicular neutrophils stained for NAP and generally more intensely, although the variation was considerable. Application of Student's 't' test to the means for the percentage positive cells and also for the scores showed no significant difference between the two cell populations ($t = 1.006$ and 1.08 respectively, $p > 0.1$). Linear regression analysis of the blood on crevicular values demonstrated no significance in either percentage positivity or total scores. As an example, Fig. 2.7 shows the results from one subject studied. The difference in distribution of staining intensity between the two cell populations within one subject is apparent, although this is not accounted for by standard statistical analysis.

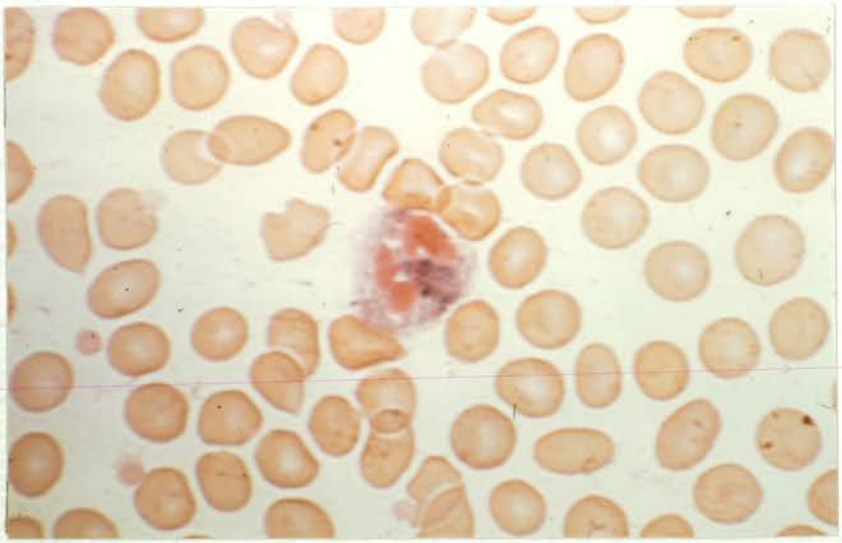


FIG. 2.6a: Peripheral venous blood film stained for alkaline phosphatase. Neutrophil shows positive staining, score 2. x 1200.

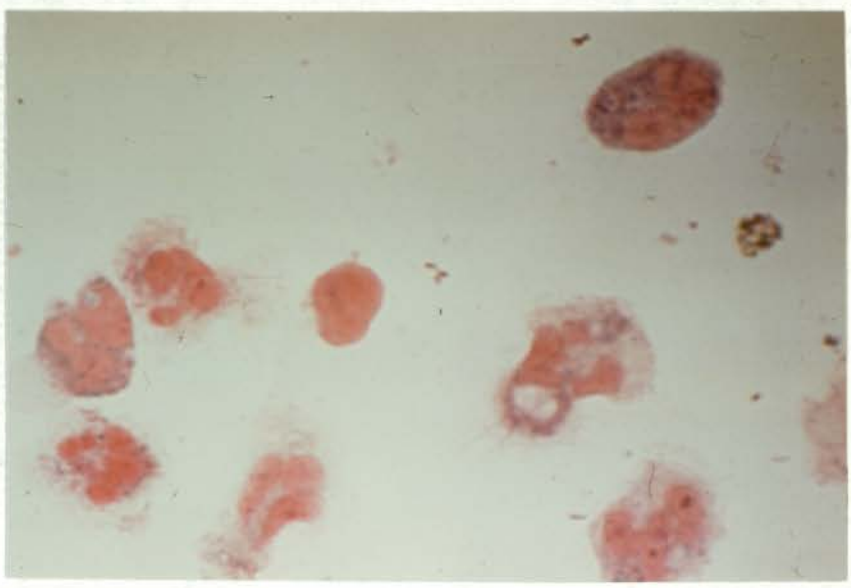


FIG. 2.6b: Crevicular neutrophils stained for alkaline phosphatase. Cells are stained to varying degrees. x 1200.

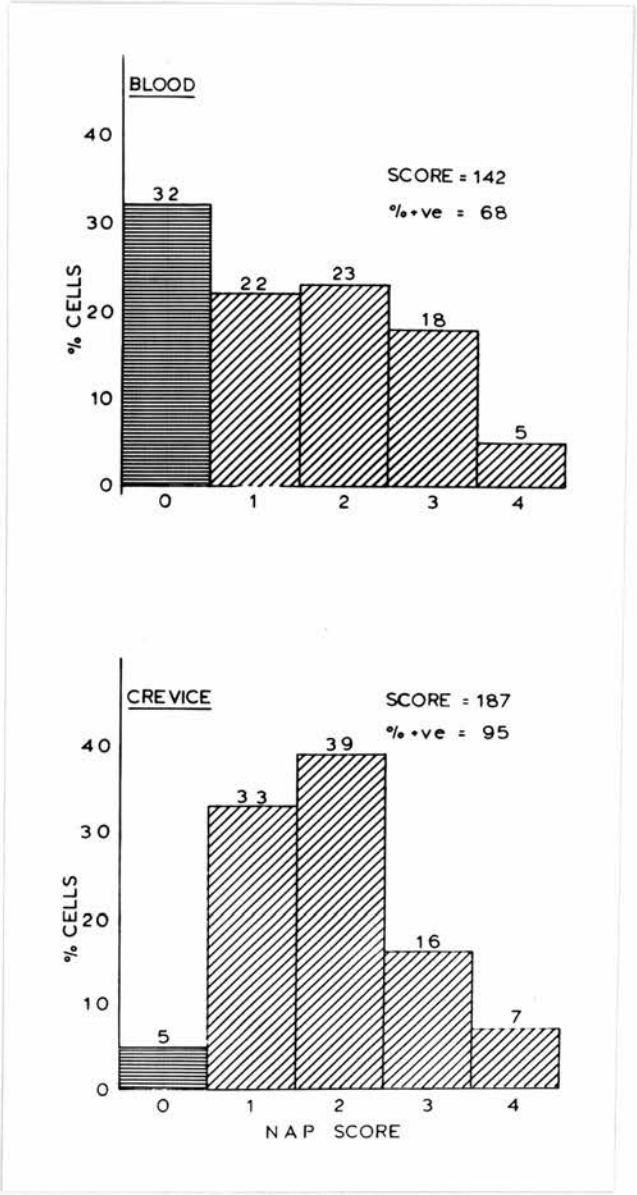


FIG. 2.7: Distribution of NAP scores for peripheral blood and crevicular neutrophils from one healthy adult male subject (age 40 years).

DISCUSSION

To minimise the theoretical risk of cross infection, it was desirable to develop a crevicular fluid collecting technique using entirely disposable materials. However, for purely technical reasons, the best cell recovery was obtained using a fixed needle 50 µl Hamilton syringe as originally described by Skapski and Lehner (1976). The design of the needle eliminates the problems of dead space and also the turbulence arising from sections of widely differing diameters, which could cause cellular distortion. The manufacturers describe specific cleaning procedures which were extended by always rinsing the syringe and plunger in disinfectant (Chloros; ICI Ltd., Birtley, Co. Durham), then strong detergent (Decon; Decon Laboratories Ltd., Hove, Sussex), followed by rinsing in water after use. By such a regime it was hoped that any risk of contamination or cross-infection would be minimised.

The viability of the majority of crevicular neutrophils as determined by Trypan blue dye exclusion confirmed previous studies on human subjects (Skapski and Lehner, 1976; Scully, 1982). The presence of clinically evident gingivitis did not influence the proportion of cells excluding dye. Skapski and Lehner (1976) reported a further increase in viability with decreasing cell yields by rinsing successively 12 times at each site. In the

present study the first two washings were discarded in order to remove the likelihood of including old, degenerating cells, but subsequent washings were not examined individually for cell viability.

One of the initial aims of this study was to explore the possibility of developing a rapid, simple screening test based on the collection of crevicular exudate neutrophils. By making glass slide preparations with the cytocentrifuge, this objective was realised, at least in part. It was clear that crevicular neutrophils are fragile, and 'gentle' centrifugation was required to preserve their morphology. Too low a speed did not provide sufficient force to place the cells onto the glass, particularly if the cycle time was short. On the other hand, too high a speed (e.g. that actually recommended by the manufacturer) resulted in the cells being 'smashed' against the glass slide and grossly distorted, irrespective of the cycle time. Thus, the conditions deemed most appropriate resulted in a centrifugation force of 14 *g* , which is considerably less than the 83 *g* used by Skapski and Lehner (1976).

Despite the apparent cell fragility described above, storage for up to 30 minutes was possible between 4°C and 37°C with no loss of morphological integrity. This was a potentially important point, if in subsequent tests the cells were to be held prior to use. Crevicular fluid

is a complex exudate containing electrolytes, ions and proteins (Cimasoni, 1974); they probably contribute to the maintenance of cellular homeostasis.

From a single cytocentrifuge preparation, three parameters were measured: the total cell count, the differential leucocyte count and in vivo phagocytosis. The neutrophil count as determined from the calculation described, although simplistic, appeared as accurate as the traditional 'wet' counting chamber method which is also error-prone. This was verified by statistical analysis. The counts reported were consistent with those of Wilton et al. (1977a,b) who reported equally large standard errors using a counting chamber. No correlation between the inflammatory state of the gingivae and the cell count was demonstrable. This confirms the unsuitability of using the migrating cell numbers as an index of clinical inflammation (Hase and Reade, 1979).

In the classical description of an inflammatory response, neutrophil granulocytes are eventually superseded, usually after some hours, by macrophages. This can be demonstrated artificially in the skin window as described by Rebuck and Crowley (1955). In the gingival crevicular exudate, however, this does not happen, and the proportion of neutrophils is consistently high. From the same Leishman stained preparations in this study, the proportion of neutrophils in crevicular

fluid washings was shown to be approximately 97%, which confirms the findings of others (Egelberg, 1963; Attström, 1970; Raeste, 1972a; Wilton et al., 1977b). Apart from the very low proportion of neutrophils reported by Wright (1968), which was based on cells collected from saliva, other studies on cells obtained directly from the crevice have put the differential neutrophil count at approximately 90% (Skapski and Lehner, 1976; Wilton et al., 1976). These workers also employed standard Romanowsky staining techniques. An explanation for the difference may be in the small proportion of neutrophils that are starting to undergo degenerative changes, seen more dramatically in salivary cells (Raeste and Calonius, 1971). Thus, these cells with rounding nuclei may be scored as mononuclear cells. In addition, epithelial cells with contracted cytoplasm, present in large numbers in the crevice, may resemble monocytes. Finally, there is the possibility that slight trauma, produced by the collecting procedure but undetected clinically, could result in additional extravasation of neutrophils and mononuclear cells. The combined method for non-specific and chloroacetate esters employed in this study served to resolve the issue. Although there is no suggestion of a statistically significant difference between the mean differential counts shown in Table 2.8 for the five subjects studied ($t = 0.18$; $p > 0.1$), it is clear that with the Leishman

staining method a proportion of neutrophils were being scored as mononuclear cells.

The conclusion to be drawn is that 'the' leucocyte of the gingival crevice is the neutrophil granulocyte. It may be hypothesised that mononuclear leucocytes are present only accidentally, perhaps as a result of chance extravasation, of microscopic tissue damage produced during food impaction, or of the micro-ulceration of chronic sub-clinical inflammation. Histological examination of gingival tissue has revealed that mononuclear leucocytes as well as neutrophils are a prominent and distinctive feature during all stages of chronic gingivitis and periodontitis. This infiltration is restricted largely to the connective tissue and to a limited extent the epithelium, but there is no evidence that the monocytes or macrophages and lymphocytes are *en passage* to the crevice, as are the neutrophils (Page and Schroeder, 1976; Page and Schroeder, 1982: 251-260).

Almost a quarter of the cells in the samples studied showed evidence of *in vivo* phagocytosis having occurred, so confirming the findings of others (Attström, 1970; Scully, 1980b). There was considerable variation both within and between individuals, but these findings support the hypothesis that crevicular neutrophils fulfil a protective role. The left-shift in blood, according to Arneth-Cooke counts, is often associated with infection

and this example may be considered analogous. Many cells had bacteria adhering to the surface or the perimeter as shown in Fig. 2.5a. Since the fate of crevicular cells is to be shed into saliva and swallowed, this phenomenon must also be considered as a non-specific, protective response. No correlation was shown between the percentage of neutrophils exhibiting *in vivo* phagocytosis and cell count for the test group, but the possibility of phagocytosis occurring is likely to be increased if the potential for bacterial-neutrophil collisions increases within certain, unknown numerical limits. Other factors such as opsonisation must also be important. This may help to explain the high proportion of cells containing bacteria in one of the children with a concomitant low crevicular neutrophil count.

Alkaline phosphatase was assayed histochemically to determine the feasibility of this type of investigation in crevicular neutrophils. The enzyme was demonstrable and more so than in peripheral venous blood neutrophils collected simultaneously. This finding is consistent with the fact that NAP is often raised (in peripheral blood cells) during leucocytosis of severe infection, among other clinical conditions. The gingival crevice is a site of active confrontation between phagocytes and bacteria, and this finding complements that of the Arneth-Cooke counts. Controversy exists as to the

physiological function of alkaline phosphatase, there being less of the enzyme in human neutrophils than in many other species (Rausch and Moore, 1975). The localisation within the cell is also disputed (Klebanoff and Clark, 1978: 48-49). Some workers have isolated it in the specific granule fraction (Bainton et al., 1971) while others claim its presence in the membrane-associated fraction (e.g. Spitznagel et al., 1974). Further controversy arises because available data suggest that NAP expression is related directly to maturation (Pedersen and Hayhoe, 1971; Scott et al., 1982) although others argue that the relationship is an inverse one (Williams, 1975; Mishler and Williams, 1980). Thus, the results presented here could be interpreted according to either hypothesis.

The NAP scoring system being semi-subjective, leads to wide variation in standard haematological practice so that control patients are always required when testing for suspected defects, as occurs classically in chronic myeloid leukaemia. The blood values obtained were comparable to those of a standard haematology laboratory (G. Stockdill, personal communication). Crevicular neutrophil values seemed to be higher than those for blood, although possibly due to the small sample number, the differences were not statistically significant.

SUMMARY

It has been demonstrated that neutrophils are readily collected in gingival crevicular washings in large numbers. From cytocentrifuge preparations carefully executed, total counts, differential counts and an estimate of phagocytic function and basic histochemical assays can be performed. The results of all these studies indicate a functional, far from expired, population of continuously migrating neutrophils that are readily available for further investigation.

CHAPTER 3

NITROBLUE TETRAZOLIUM REDUCTION BY BLOOD AND GINGIVAL CREVICULAR NEUTROPHILS: THE REDUCING ACTIVITY OF SALIVA

GENERAL INTRODUCTION

Circulating neutrophils are in transit between their production site in the bone marrow and functional challenge sites in the tissues, exudates and secretions. In addition to the 'physiological' role, there clearly exists, in terms of number, an enormous phagocytic cellular potential to meet the demands of a localised or systemic microbial invasion. Unless confronted by some particulate or soluble antigenic stimulus, neutrophils exhibit little metabolic activity, having very few mitochondria, sparse endoplasmic reticulum and being incapable of mitosis (Klebanoff and Clark, 1978: 191-192). Each cell, however, has an enormous reserve and stimulation of the cell membrane, which may be the result of phagocytosis, produces an outstanding increase in metabolic activity, and a concomitant increase in cellular oxygen consumption. It is this 'metabolic burst' or 'respiratory burst' that is central to subsequent microbicidal function, via the so-called oxygen-dependent systems.

Neutrophil Metabolism

The neutrophil performs a range of functions which require it to produce and expend energy, for example cellular adherence, chemotaxis, phagocytosis and any membrane mobilising activities. As stated above, few mitochondria are seen in mature neutrophils and thus oxidative phosphorylation accounts for a small proportion of ATP production, over 99.9% being produced by anaerobic glycolysis (Sbarra and Karnovsky, 1959; Jemelin and Frei, 1970; Stjernholm and Manak, 1970; Klebanoff and Clark, 1978: 339). During glycolysis, via the Embden-Meyerhof pathway there is a net production of two ATP molecules (four less two utilized in phosphorylation reactions) resulting in the generation of lactate. The lactate may contribute to bactericidal systems either directly, or indirectly by reducing the pH in the phagolysosome (Hirsch and Cohn, 1960). Some authors have recently suggested, however, that this is of little significance (Murphy, 1976: 171; van Zwieten et al., 1981).

The principal energy source is thus glucose or its storage form glycogen, which comprises 1-2% of the wet weight of the human neutrophil (Klebanoff and Clark, 1978: 327). Glucose, whether exogenous in origin or endogenous from glycogenolysis, is degraded anaerobically to pyruvate and lactate. Following glucose-6-phosphate formation, the pathway may be direct glycolysis to

lactate or via the hexose monophosphate shunt (HMPS). The HMPS, or pentose cycle, is a series of reactions by which pentose sugars are produced as the basis of nucleotide synthesis or more importantly in the case of the neutrophil, to be returned to the glycolytic pathway. Of vital importance, however, is that during this process the cell generates NADPH, essential for reducing reactions central to microbicidal systems (Fig. 3.1).

There is thus a crucial link between energy metabolism and the metabolic 'diversion' created for the specific functions of this cell.

The HMPS is dependent upon the availability of $NADP^+$, generated from, for example, the glutathione system (Fig. 3.1). In the resting state glucose utilisation via the HMPS accounts for only 5% of the total (Beck, 1958a; Stjernholm and Manak, 1970) whereas with phagocytosis this rises to nearly 40% (Stjernholm and Manak, 1970). By contrast, direct glycolysis predominates as the major pathway in the resting cell (Beck, 1958b) and increases only slightly during phagocytosis (Stjernholm and Manak, 1970). Inhibition of the glycolytic pathways results in inhibition of phagocytosis (Elsbach, 1972). Phagocytosis proceeds normally in anaerobic conditions although bacterial killing is impaired (Selvaraj and Sbarra, 1966; Mandell, 1974). Complete microbicidal

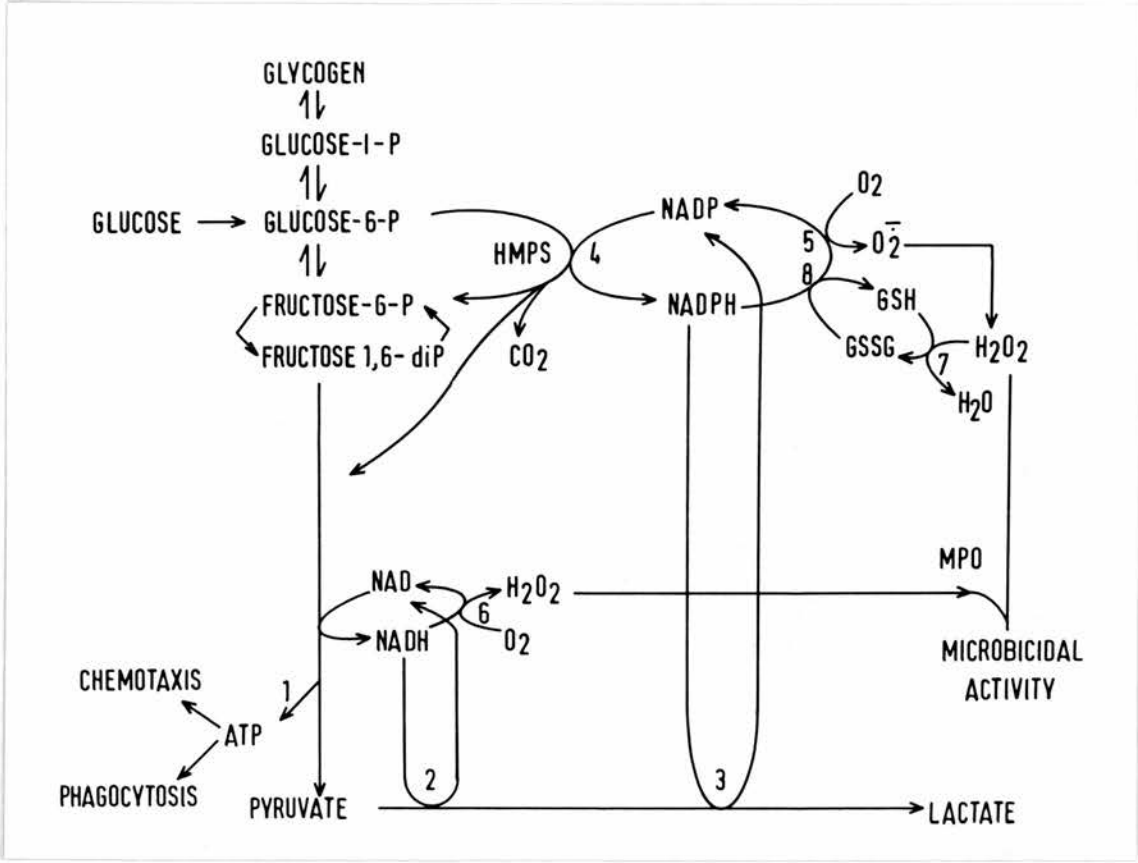


FIG. 3.1: The link between glycolysis, HMPS, the generation of NADP and the initiation of the oxygen-dependent microbicidal pathway. Adapted from Klebanoff and Clark, 1978: Figs. 6.11, 6.13 and 6.19.

Key to enzymes:

1- Pyruvate kinase
2- NADH-linked lactate dehydrogenase
3- NADPH-linked lactate dehydrogenase
4- Glucose-6-phosphate dehydrogenase (and then 6-phosphogluconate dehydrogenase)
5- NADPH oxidase
6- NADH oxidase
7- GSH peroxidase
8- GSSG reductase

integrity depends upon an intact oxygen-dependent system which, as stated above, is crucially linked to the HMPS. The enzyme which forms this link via which molecular oxygen is reduced to water, is the membrane-associated oxidase. This will be considered in the next section.

The Metabolic Burst

Fifty years ago it was observed that canine neutrophils challenged with Sarcina lutea exhibited an increase in oxygen consumption (Baldridge and Gerard, 1933) and this phenomenon has been confirmed by other groups of workers (Sbarra and Karnovsky, 1959; Cohn and Morse, 1960). Non particulate stimuli which are surface active, also produce this effect, but without a classical phagocytic response (Goetzel and Austen, 1974; Schell-Frederick, 1974; Rossi et al., 1977). The increase in oxygen uptake is extremely rapid, occurring within seconds of membrane stimulation (Patriarca et al., 1971; Rossi et al., 1972; Segal and Coade, 1978) and is sustained for several minutes (De Chatelet et al., 1972; Segal and Coade, 1978; Romeo et al., 1979). The subsequent fate of the oxygen is entirely dependent on initial triggering of the neutrophil membrane, and the associated activity of an oxidase enzyme (De Chatelet, 1978). Oxygen consumption is governed to some extent

by the nature of the stimulus (Klebanoff and Clark, 1978: 284), but it is unaffected by cyanide, so demonstrating that the cytochrome system is not responsible for the metabolic burst (Sbarra and Karnovsky, 1959). The consensus view is that the key enzyme is an NADH or NADPH oxidase and intense efforts have been directed towards identifying its precise localisation (see reviews by Karnovsky, 1973; Klebanoff, 1975; De Chatelet, 1978).

Over the past few years several neutrophil enzymes, such as myeloperoxidase, NADPH oxidase, NADH oxidase and amino acid oxidase, have been proposed as candidates for the oxidase (Klebanoff and Clark, 1978: 352-361). Klebanoff and Clark (1978) clearly favoured either myeloperoxidase or an NADPH oxidase. In their review, they stated "Conclusive evidence for the presence of a distinct NADPH oxidase would be its purification and the elucidation of its structure and properties. This has not yet been reported." (Klebanoff and Clark, 1978: 361). It should be repeated that whatever the nature of the oxidase, it must provide the essential link with the activation of the HMPS, and Klebanoff and Clark (1978: 362) stated that NADPH oxidase would meet these criteria.

Research activity into the nature, localisation, properties and function of the NADPH oxidase has been intensive over the past few years (Babior, 1983). There

are various reasons for the degree of interest in this single aspect of phagocytic cell function, among which are the relevant ease of cell collection and functional investigations, but more importantly there is a clear observable relationship between a molecular and biochemical function, as well as dysfunction producing dramatic clinical disease. The rare hereditary and debilitating chronic granulomatous disease (CGD) and leucocyte glucose-6-phosphate dehydrogenase deficiencies are directly related to dysfunction in the NADPH oxidase and related HMPS.

Although Iyer and Quastel (1963) described the principle of the oxidase reaction, it transpired that they were probably measuring myeloperoxidase activity, since they studied resting cells. NADPH oxidase only becomes stimulated in conjunction with the HMPS on stimulation of the neutrophil. The actual identification was made in a series of studies by Rossi and his colleagues (Rossi and Zatti, 1964; Patriarca et al., 1971; Patriarca et al., 1975; Romeo et al., 1979), and their findings have been confirmed by other workers (De Chatelet et al., 1975; Babior et al., 1976; Suzuki and Kakinuma, 1983). The oxidase also appears to require specific divalent cations for optimum function (Green et al., 1983). The essential function of the oxidase is to catalyse the reaction:

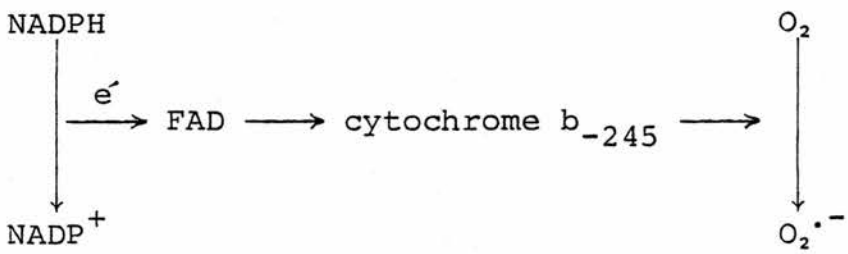
$$NADPH + 2O_2 \longrightarrow NADP^+ + 2O_2^{\cdot -} + H^+$$

By further reduction, this ultimately results in the production of water when the cell is stimulated with an appropriate agent (Babior et al., 1976; Suzuki and Lehrer, 1980).

Various studies have indicated that the NADPH oxidase is concentrated within the plasma membrane of the neutrophil (Briggs et al., 1975; De Wald et al., 1979; Badwey et al., 1980) and that, following stimulation, it can also be detected in the membranes of the phagolysosomes both in guinea pig (Bellavite et al., 1982; Tsunawaki et al., 1983) and human neutrophils (Borregaard, Heiple et al., 1983). This phenomenon incidentally is not limited to eukaryote cells (Dechartellier et al., 1983).

The precise composition of the NADPH oxidase has not been finally elucidated, partly due to technical difficulties, so that most of the information collected has been based on studying the effects and properties of the oxidase system. Considerable interest and intense study have been applied to a few putative components. Foremost of these is a low potential cytochrome b, present in plasma membranes of human neutrophils, but absent from neutrophils of patients with chronic granulomatous disease (Segal and Jones, 1978, 1980; Cross et al., 1981). Segal's group has in fact done most to elucidate many of

the properties of this unusual cytochrome and a recent paper has reviewed the state of knowledge (Segal, 1983). The cytochrome, which is only found in phagocytic cells (Segal, Goldstone et al., 1981), has the lowest mid-point potential at -245 mV of any mammalian cytochrome so far studied (Cross et al., 1981). Evidence has also been presented for the participation of a flavin, specifically FAD (Babior and Peters, 1981; Cross et al., 1982). Segal (1983) thus believes that the cytochrome b_{-245} is the terminal component of the electron transport chain which results in the reduction of molecular oxygen to the superoxide anion, and his group have proposed the system to operate as follows:



(Cross et al., 1982)

Babior (1983), however, argues against the involvement of the b cytochrome, suggesting that the kinetics of its reduction are inconsistent with those of superoxide production in human cells. He favours the participation of a quinone, quoting the evidence of other workers (Sloan et al., 1981; Crawford and Schneider, 1982). Segal (1983) does not share this view and experimental

evidence has been presented to suggest that neutrophil ubiquinone is present in the small mitochondrial fraction, and thus is not a participant in NADPH oxidase function (Cross et al., 1983). Clearly the question of precise composition remains to be totally clarified, but there is general agreement, as stated previously, that NADPH is the superoxide-generating electron donor as simply depicted in Fig. 3.1. It is also supposed that the ensuing reduction of oxygen is the basis for the oxygen-dependent microbicidal system (Babior et al., 1975; Klebanoff, 1975). Thus a number of ionic species and radicals participate directly or indirectly in bringing about bacterial killing by some form of oxidation process. Superoxide anion, the perhydroxyl radical, the hydroxyl ion, various singlet oxygen species, hydrogen peroxide and myeloperoxidase-mediated oxidations are all implicated and illustrated in Fig. 3.2 (for review see Klebanoff and Clark, 1978: 409-446). The myeloperoxidase system will be the subject of detailed discussion in chapter 4 but is dependent upon a supply of substrate, principally hydrogen peroxide, which is probably generated in part from the superoxide anion.

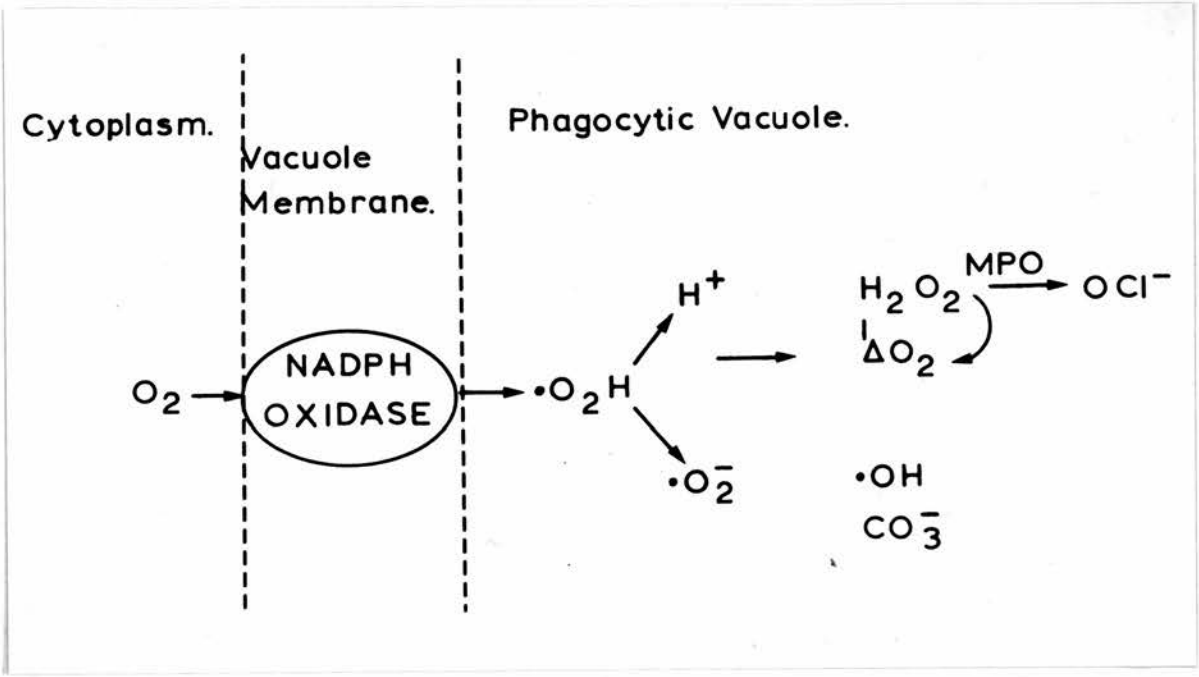


FIG. 3.2: Several radicals and ionic species are produced in the phagocytic vacuole as a result of the membrane-associated reduction of molecular oxygen.

Superoxide Anion

The first reduction product of electron transfer to oxygen is the perhydroxyl radical ($HO_2^{\cdot}$) or its ionised state the superoxide anion ($O_2^{\cdot -}$). The pKa of the reaction

$$HO_2^{\cdot} \rightleftharpoons H^+ + O_2^{\cdot -} \text{ is } 4.8,$$

so that at neutral pH the superoxide anion predominates (Klebanoff and Clark, 1978: 289). This highly reactive anion may be formed by a large number of biochemical mechanisms (Fridovich, 1972) but was first shown to be produced in human neutrophils by Babior and his colleagues (1973). In fact, nearly 30 reaction mechanisms have been proposed as resulting in superoxide production and at least a third of these may be feasible in neutrophils (Klebanoff and Clark, 1978: 290). The generation of perhydroxyl rather than superoxide has recently been proposed (Allen, 1979) but refuted by Roos, Hamers et al. (1983). The superoxide anion may of course either lose an electron and act as a reductant so being converted to oxygen, or gain an electron so acting as an oxidant and itself being reduced to hydrogen peroxide (Klebanoff and Clark, 1978: 289). Furthermore, the interaction of two superoxide ions leads to a dismutation reaction:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow O_2 + H_2O_2$$

which proceeds rapidly under the influence of the enzyme superoxide dismutase (Fridovich, 1972; 1975). There are distinct forms of dismutases varying in the divalent ion metallic content (Klebanoff and Clark, 1978: 291-293), and these are widespread in aerobic mammalian cells. It has been suggested that the principal functions of the dismutase, rather than the generation of hydrogen peroxide per se, are to protect the cell against the toxic effects of oxygen radicals (McCord et al., 1971), a concept that is receiving close scrutiny at present.

The production of superoxide by human and animal neutrophils has been studied extensively and shown to be markedly increased by the phagocytosis of latex particles (Babior et al., 1973), bacteria (Curnutte and Babior, 1974) and zymosan (Rosen and Klebanoff, 1976). In addition, soluble stimuli which perturb the neutrophil membrane, may also induce superoxide production. Examples of these are fluoride (Curnutte and Babior, 1975) and phorbol myristate acetate (De Chatelet et al., 1976). There is evidence that a significant proportion of the oxygen consumed on cellular stimulation is converted to superoxide (Curnutte and Babior, 1974; Weening et al., 1974), although its actual presence in phagocytic vacuoles is speculative. The acidic pH of the vacuole would favour dismutation to hydrogen peroxide, particularly in the presence of superoxide dismutase.

In one study approximately 50% of the hydrogen peroxide produced during phagocytosis was shown to be derived from the dismutation of superoxide (Root and Metcalf, 1977). More recently, the generation of superoxide in neutrophils has been in dispute, in the sense that it may not be an invariable product of electron transport to molecular oxygen. The grounds for controversy are based on essentially technical considerations. The standard method by which superoxide is assayed depends upon the reduction of ferricytochrome c by whole cells, and the inhibition of this reaction by superoxide dismutase (Babior et al., 1973). The principle of the reaction is as follows:

$$O_2^{\cdot -} + \text{Cyt c } (Fe^{3+}) \longrightarrow O_2 + \text{Cyt c } (Fe^{2+}),$$

so that oxygen is regenerated. In 1979, two conflicting reports were published, one of which upheld this reaction (Babior, 1979) and one which reported no such activity in stimulated neutrophils (Segal and Meshulam, 1979). The nature of the stimulant is of prime importance. It has been shown that latex is a poor generator of superoxide (Weening et al., 1975; Curnutte and Tauber, 1983), although the respiratory burst is activated (Curnutte and Tauber, 1983). In another report the soluble stimulant, N-formylmethionyl-leucyl-phenylalanine (FMLP), was far more effective in stimulating neutrophils to produce superoxide than opsonised zymosan (West et al., 1983).

These authors also suggested that other mechanisms may exist by which ferricytochrome c is reduced. Clearly, *in vivo* and *in vitro*, different stimuli may differentially trigger the univalent reduction of oxygen to superoxide or a two electron donation to produce hydrogen peroxide (Curnutte and Tauber, 1983). This view is shared by others who believe that the physiological end product of the oxidase system is hydrogen peroxide and not superoxide or the perhydroxyl radical (Roos *et al.*, 1982; Roos, Hamers *et al.*, 1983).

Measurement of Superoxide Anion

As stated above, the standard assay for superoxide depends upon the reduction of ferricytochrome c and more importantly, given the possible reduction by agents other than superoxide, the inhibition of this reduction by extraneous superoxide dismutase (Babior *et al.*, 1973; Weening *et al.*, 1975; Babior and Cohen, 1981). As with other such measurements of neutrophil function, superoxide production by stimulated or phagocytosing neutrophils is markedly greater than that for resting cells (Klebanoff and Clark, 1978: 297-298). The generation of superoxide anion may be monitored over a fixed time period or continuously, but it has been pointed out that the ferricytochrome c does not enter the cells. Therefore,

these assays only detect the superoxide which is released into the extracellular medium, with the result that an unknown proportion may be undetected (Babior and Cohen, 1981). An indirect assay of superoxide production is possible by utilising the intracellular reduction of a soluble dye, nitroblue tetrazolium (NBT).

Nitroblue Tetrazolium Reduction

The historical development of tetrazolium salts and their applications in oxidoreductase histochemistry have been described in detail by Pearse (1972: 880-920). The first compound, prepared almost 90 years ago, was triphenyl tetrazolium chloride, and subsequent development through the first half of the 20th century was slow. However, after the Second World War, there was a demand for more sensitive agents and a second group of tetrazolium agents became available, neotetrazolium, blue tetrazolium and iodophenyl-nitrophenyl substituted tetrazolium chloride. These were quickly applied to histochemical methods and widely used for nearly ten years. One of these, neotetrazolium chloride, was used in a series of sophisticated experiments to determine the metabolic response of rat peritoneal exudate cells (60-70% neutrophils) to brucella organisms (Shaffer et al., 1953). These workers were not able to use nitroblue tetrazolium,

as stated erroneously by Klebanoff and Clark (1978: 308), because it was developed along with others in the next generation of compounds, between 1956 and 1963 (Pearse, 1972: 882).

Other compounds have since followed in attempts to increase sensitivity and quality of staining, which depend on a number of factors, particularly the redox potential, substantivity* for protein and lipid solubility. The half-wave potential of a tetrazolium salt indicates the theoretical ease of reduction to a coloured formazan, so that nitroblue tetrazolium with an $E_{\frac{1}{2}}$ value of -50 mV, at pH 7.2, is easily reduced and particularly useful in biological systems, since its other properties are also favourable. NBT is a substantive ditetrazole, and more fully described as 2,2'-di-p-nitro-phenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride (Tsou et al., 1956).

The reduction of a tetrazolium salt involves the acceptance of electrons from an oxidised substrate or intermediate electron carrier with a conversion to a coloured formazan. In dehydrogenase histochemistry, under the influence of a tetrazolium reductase or diaphorase (Pearse, 1972: 907), the reaction would be:

$$\text{NAD(P)H} + \text{tetrazolium salt} \rightleftharpoons \text{NAD(P)}^{+} + \text{formazan}$$

Unless there is depletion of the pyridine nucleotide via

*Affinity of the tetrazolium salt as defined by chemical thermodynamics.

a different pathway, this reaction will proceed to the right, since formazans are not reoxidised in biological systems. As Pearse (1972: 917) states, given the relative redox potentials of the biological mechanisms under investigation and the tetrazolium salts, particularly those more recently developed, there is every reason to suppose that electrons will flow from the former to the latter. With manipulation of this chemistry, and choosing an appropriate combination of other properties in the system, tetrazolium salt reduction has been applied to the intersection of electron transfer in plant tissues as well as prokaryotic and eukaryotic cells.

Nitroblue Tetrazolium Reduction in Neutrophils

Neutrophil leucocytes, particularly stimulated cells, clearly possess the potential for reduction of tetrazolium salts. Shaffer and his colleagues (1953) demonstrated this with neotetrazolium (NT) and similar results were subsequently published to confirm their findings, and all demonstrated that the neutrophil leucocyte was the most reactive of all human peripheral blood cells (Marcuse and Cochran, 1961). However the newer nitroblue tetrazolium (NBT) offered increased sensitivity (for example, $E_{\frac{1}{2}}$ NBT - 50 mV, NT - -170 mV) with the production of an intense blue or black formazan.

In 1960 Quaglino and Hayhoe employed an acetone fixation method with NBT to demonstrate dehydrogenases in leucocytes, but one year later a method was described using NBT in a supra-vital system (Balogh and Cohen, 1961). In 1966, Baehner and Nathan reported a lack of NBT reduction by neutrophils from patients with CGD and published a more complete account the following year (Baehner and Nathan, 1967). This heralded a period of intense interest and activity in clinical applications of NBT testing. Initially a number of workers confirmed the results of Baehner and Nathan (Windhorst et al., 1967; Park et al., 1969; Gifford and Malawista, 1970), but attention soon focused on the possibility of using NBT reduction as a marker for bacterial infection (Park et al., 1968). A number of studies were encouraging (Freeman and King, 1972a; Wollman et al., 1972; Freeman et al., 1973), but others drew attention to difficulties (Matula and Paterson, 1971; Gordon et al., 1973) and considered the test unreliable (Segal et al., 1973; Segal, 1974; Steigbigel et al., 1974). Subsequently a series of reviews has been published in an attempt to provide a balanced appraisal of NBT tests (Editorial, Lancet, 1971; Gordon and Rowan, 1973; Segal, 1974; Ward, 1974; Klebanoff and Clark, 1978: 308-316).

Many potential problems have been discussed in these reviews. For example the anticoagulant used following blood

collection may influence the results (Gordon et al., 1973; Hellum and Solberg, 1973; Björkstén, 1974). The concentration of NBT used in a test may be sufficiently high as to be toxic to the neutrophils (De Chatelet and Shirley, 1975). Drug therapy may influence the neutrophil response. For example some authors have reported that steroids depress NBT reduction (Mandell et al., 1970) whilst others have found no effect (Wollman et al., 1972). With respect to using NBT tests for diagnosing clinical infections, extensive lists have been produced documenting situations in which both false negative results (normal or low values in the presence of bacterial infection) and false positive results (increased values in the absence of infection) may occur. Klebanoff and Clark (1978: 314-315) cite 11 circumstances in which the former may be seen and 31 of the latter. Current practice with respect to the diagnosis of bacterial infection is to use an NBT test only in conjunction with other investigative procedures. It has been emphasised that there is no such thing as the NBT test (Freeman and King, 1975), and each laboratory must establish a method and determine normal values and ranges for that method. In clinical immunology the test is, however, invaluable for diagnosing the existence of CGD and this will be discussed further in chapter 5. When CGD is suspected, the so-called "stimulated" NBT test, devised by Park and Good (1970) is virtually confirmatory.

Unstimulated neutrophils from a healthy donor with no bacterial infection reduce NBT to a very limited extent. Positive cells occur for a number of reasons. For example NBT may diffuse through the plasma membrane, or heparin-formazan complexes may be formed which can be phagocytosed (Segal and Levi, 1973). Metabolic stimulation may occur as a result of the test system, e.g. when cells are adherent to glass (Gifford and Malawista, 1970). Park and Good's stimulated test was designed to override these factors by producing a definitive challenge to the cells, which is clearly seen in normal neutrophils and clearly absent in neutrophils from a patient with CGD.

After Baehner and Nathan (1966) triggered interest in NBT tests, they have been developed broadly along two lines. Semi-quantitative histochemical techniques as first described by Park et al. (1968) employ an incubation of cells with NBT, followed by glass slide preparation, so that the percentage of positive cells can be counted microscopically. This approach is technically simple, but care is needed to avoid cell clumping during incubation (Ward, 1974). Many variations of this test and modifications have been described (Preisig and Hitzig, 1971; Freeman and King, 1972b; Gordon et al., 1973; Gordon et al., 1975; Babior and Cohen, 1981). A method which permits the use of small cell numbers was described by Gifford and Malawista (1970). As mentioned above, the

cells are permitted to adhere to a glass slide before being challenged and variations on this technique have also been published (Gifford and Malawista, 1972; Repine et al., 1979; Babior and Cohen, 1981).

The other technique is spectrophotometric and is therefore fully quantitative (Baehner and Nathan, 1968). This provides a precise measure of the total NBT reduction by a given number of cells, but requires more sophisticated equipment. Recently a miniaturised quantitative test was described, which permits the rapid processing of multiple tests (Hofstaetter and Brammsen, 1981; Pick et al., 1981).

Since the initial promise of a universally acceptable NBT test for monitoring and diagnosing infection was not fulfilled, the review articles of the mid-1970s were followed by a much reduced rate of publication on this subject, largely devoted to laboratory research, and in particular the reduction mechanism.

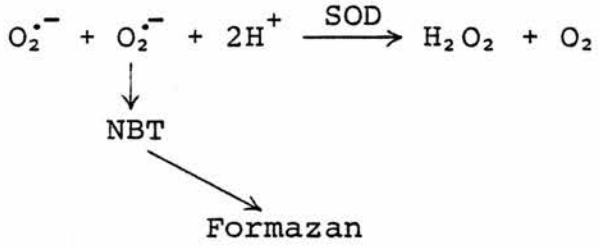
The principle of NBT reduction is common to all these techniques and variations, and any established test should demonstrate an increase in reduction by normal neutrophils when stimulated.

Mechanism of Nitroblue Tetrazolium Reduction by Neutrophils

Over the past 15 years uncertainty has prevailed as to the precise mechanism of NBT reduction in neutrophils. It has always been apparent that the source of electrons which effect the reduction must centre on the membrane oxidase, and thus the metabolic or respiratory burst. The discussion and debate as to the nature of that oxidase has been described earlier in this chapter.

The formazan of reduced NBT first appears in the phagocytic vacuoles and later in the cytoplasm (Nathan et al., 1969). The associated cyanide-insensitive enzyme or enzymes can be isolated from both the soluble and membrane fractions of neutrophils (Humbert et al., 1973; De Chatelet et al., 1974; Segal and Peters, 1977). Over ten years ago it was demonstrated that solubilised fractions of human neutrophils contained several NBT-reducing pyridine nucleotide diaphorases (Holmes and Good, 1972), and recent studies using polyacrylamide gel electrophoresis have confirmed this (Phillips et al., 1982). These latter authors point out that this approach is limited in that the considerable membrane-associated enzyme activity is lost, as may be enzyme co-factors, as a result of the experimental procedure.

From the studies on cell sonicates it would appear that a direct reduction of NBT is possible by reduced NAD or NADP and in disrupted cells may therefore be oxygen independent. However, in intact human cells there appears to be an absolute requirement for oxygen and Baehner's group have proposed a scheme for NBT reduction which depends upon a functional respiratory burst oxidase (Baehner et al., 1975).



SOD = superoxide dismutase

They also showed the NBT reductase activity to be located in the microsomal cell fraction (Baehner, 1975). Oxygen is as essential for the operation of this mechanism as for HMPS activity, hydrogen peroxide production and iodinization.

NBT can be reduced directly by the superoxide anion (Rajogopalan and Handler, 1964; Beauchamp and Fridovich, 1971) and very efficiently with an intermediate electron carrier (Nishikimi et al., 1972). Indirect evidence comes from the observation that superoxide dismutase blocks NBT reduction (Curnutte and Babior,. 1974) although this activity may be less specific (reviewed by Klebanoff and Clark, 1978:

309). There is ample evidence to support the contention by Baehner's group (Baehner et al., 1975) that superoxide anion generation is essential for NBT reduction in vivo. This is perhaps an illustration of a controversy brought about by extrapolation of data obtained from separated enzyme fractions and comparing this to experiments on whole cells. The evidence from each side is impressive, remembering that there is a long tradition of using tetrazolium salt reduction in dehydrogenase histochemistry (Pearse, 1972). The two hypotheses are not however mutually exclusive, as Klebanoff and Clark (1978: 309) have concluded in their presentation of a reaction for NBT reduction:

$$NAD(P)H + O_2 \xrightarrow{\text{Flavoprotein}} NAD(P) + O_2^{\cdot -} + H^+$$

$$O_2^{\cdot -} + NBT \longrightarrow O_2 + \text{Formazan}$$

This is depicted in an expanded form to indicate the metabolic events in Fig. 3.3.

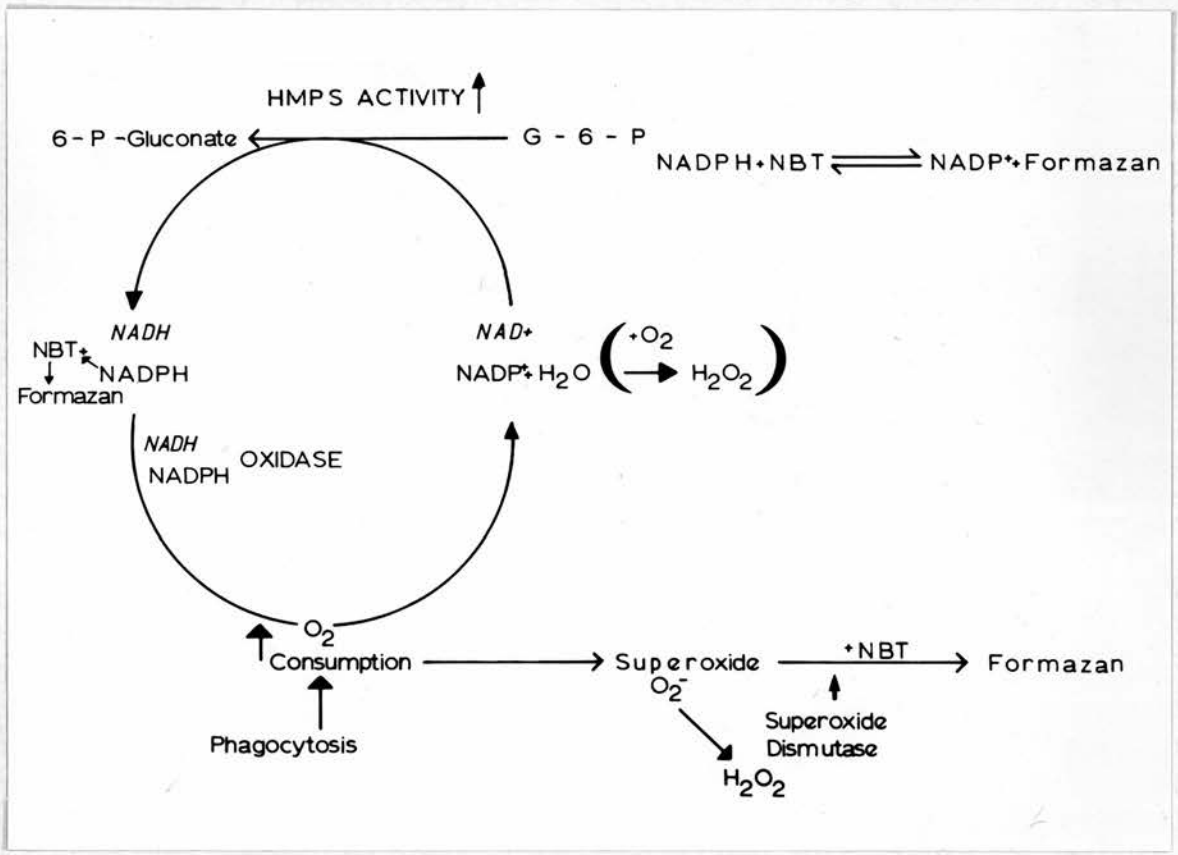


FIG. 3.3:

Mechanisms whereby NBT may be reduced to its formazan.

The Present Study

Nitroblue tetrazolium reduction must be seen as an indirect assay of the respiratory burst linked with oxygen-dependent microbicidal systems. At the time of starting this work, there were no data regarding the NBT reducing activity of exudate neutrophils, and no metabolic studies of any kind on gingival crevicular cells. One study, published in Japanese, had reported reduced NBT reduction by peripheral blood neutrophils in patients with periodontal disease and concluded that this was the result of some suppressive serum factor (Kusunoki, 1977).

The conditions under which an NBT test are conducted must be standardised by each laboratory. In this study a suitable test method for peripheral blood neutrophils was established first, so that the results obtained could be compared with those published by other workers. Having developed a test for blood neutrophils and demonstrated its integrity in a "stimulated" system, it was then possible to proceed to a method for gingival neutrophils. Finally, a chance observation during the course of these experiments revealed the strong NBT-reducing property of whole human saliva which contains large numbers of neutrophils, and this was also investigated.

NITROBLUE TETRAZOLIUM REDUCTION TEST FOR PERIPHERAL BLOOD NEUTROPHILS

Introduction

Initially several attempts were made to establish a "glass-adherent cell" type of test based on the method of Gifford and Malawista (1970). It proved difficult to obtain satisfactory preparations of formazan-containing neutrophils and therefore an alternative approach was tried.

In routine haematological screening of patients, 2.5 ml of peripheral blood are collected in a plastic tube anticoagulated with ethylenediamine tetra-acetic acid (EDTA) (Stayne Laboratories Ltd., England), and an automated system is used for cell counting and determining haematological indices. For convenience and economy, it was therefore proposed to use these tubes for the histochemical NBT test, similar in principle to that of Park et al. (1968). A low molecular weight dextran solution was incorporated in the reaction for two reasons. Firstly, some erythrocyte sedimentation would occur, thus giving a "cleaner" preparation of leucocytes, which should not require further purification for the purpose of this test. Secondly, Freeman and King (1972c)

had stated that a whole blood method is more reliable than one in which a full separation is performed, but that EDTA as anticoagulant produces low and inconsistent results. Stuart and Simpson (1970) however, showed that a sucrose polymer, Ficoll, would reverse the cell membrane lesion induced by the EDTA, and this was confirmed by others (Gordon et al., 1973). A dilute dextran/sugar solution might be supposed to have a similarly restoring effect on the neutrophil membranes.

Materials and Methods

As a starting point for the development of the test, peripheral venous blood (PVB) was collected from healthy young volunteers (both male and female aged 20-35 years).

The NBT used throughout all experiments was that produced by Sigma Chemical Co. Ltd., USA. Each vacuum sealed phial (product code 840-10) contained 1 mg nitroblue tetrazolium lyophilised with phosphate buffer and sodium chloride. This was reconstituted by the addition of 1 ml distilled water, with a resultant pH of 7.2.

A commercially available preparation of low molecular weight dextrans in sugar was employed. "Lomodex 70" (Fisons Ltd., England) comprises 6% w/v dextrans in 5% w/v

dextrose for injection. This was supplied in sterile containers, but being slightly viscous was diluted 2:1 with buffered sterile saline, pH 7.2, before use.

In summary, the basic test was performed by collecting 2.5 ml peripheral venous blood in EDTA-coated plastic tubes, to which were added 2 ml saline-dextrose solution (sds) and 1 ml NBT solution containing 1 mg NBT. These were thoroughly mixed and the tube incubated at 45^0 to the vertical under various conditions subsequently to be described. Following incubation, 0.1 ml aliquots of the supernatant fluid were placed in wells of the cytocentrifuge as detailed in chapter 2, and glass slide preparations made from which a semi-quantitative assay of NBT reduction was made. Each test was performed in duplicate and five counts of 100 neutrophils made per slide (at x 500 magnification with oil immersion), so that ten counts were made in total from which a mean was calculated. The results were expressed as a percentage of positive cells.

Based on the literature describing NBT test methods, some aspects of the test conditions had to be determined, while others were predetermined. The time of incubation, the medium of incubation (air or water), the effect of storing the blood samples, the most appropriate level at which to remove cells for centrifugation after incubation and, finally, testing the method in a "stimulated"

system, all had to be investigated. From many published methodologies already reviewed, it was clear that the temperature of incubation should be 37°C for several reasons. It is physiological and the reaction is more likely to proceed at an acceptable rate than at room temperature (generally around 19-23°C in a laboratory). Also, using a laboratory incubator, the temperature can be fully controlled, whereas this is not possible on a laboratory bench. The final concentration of NBT in the above described system was 1 mg in a total volume of 5.5 ml, i.e. 0.18 mg/ml. This was in accordance with other methods (Segal, 1974; Ward, 1974) and was therefore adopted as standard.

Time of incubation

It was clear from the literature that a standard incubation time for this type of experiment is 30 min. The test was conducted as described above with incubation at 37°C in air for 20 min, 30 min and 40 min. Peripheral venous blood was taken from three healthy adults and tested within half an hour of venepuncture.

Incubation in air and water

Björkstén (1974) suggested that a water bath is preferable for incubation since the test sample equilibrates to 37°C more quickly than in air. This was

examined by performing the above test on two samples from one healthy subject in the same incubator simultaneously. One sample was placed in air, while the other was placed in a 37°C water bath within the incubator.

The effect of storage on blood samples

For many routine haematological assays, blood samples that cannot be processed immediately are stored at 4°C. Björkstén (1974) also found this to be the optimum storage temperature for subsequent NBT testing of cells.

PVB was taken from each of two healthy volunteers and divided into seven 2.5 ml aliquots in EDTA tubes as before. One sample was processed immediately as above and the remaining six were divided into two groups. Three tubes were left on the laboratory bench (23°C) and three were placed at 4°C in a refrigerator. Tests were subsequently conducted on these at 2, 4 and 8 hour intervals.

Effect of sedimentation on NBT reduction

After 30 min incubation, despite the use of a relatively low molecular weight dextran solution, significant erythrocyte sedimentation always occurred, thus facilitating the counting of neutrophils on the

slide preparations. The two 0.1 ml aliquots taken following incubation were routinely removed, one from the upper and one from the lower level of the supernatant region. No differences were apparent in the activity of neutrophils from these two sites, but an experiment to test this possibility was performed on samples from three subjects. Figure 3.4 illustrates the four levels from which 0.1 ml aliquots were removed, following 30 min incubation at 37°C.

Application of stimulants to NBT tests

Finally, it was essential to establish that the test now devised would function as required when a stimulant was applied to the neutrophils. Several bacterial species and a commercial endotoxin preparation (Sigma Chemicals, USA) were tested against blood samples from six healthy adult volunteers. The test was modified by replacing 0.5 ml of the saline-dextrose solution during the incubation with 0.5 ml suspension of bacteria, at an original concentration of 1×10^8 organisms per ml as estimated turbidometrically and checked by viable counting. The final concentration of bacteria was therefore approximately 9×10^6 per ml. None of the organism samples was preopsonised for these experiments. The endotoxin was reconstituted as recommended by the manufacturer with distilled water, and 0.5 ml of the resultant solution substituted for the bacteria.

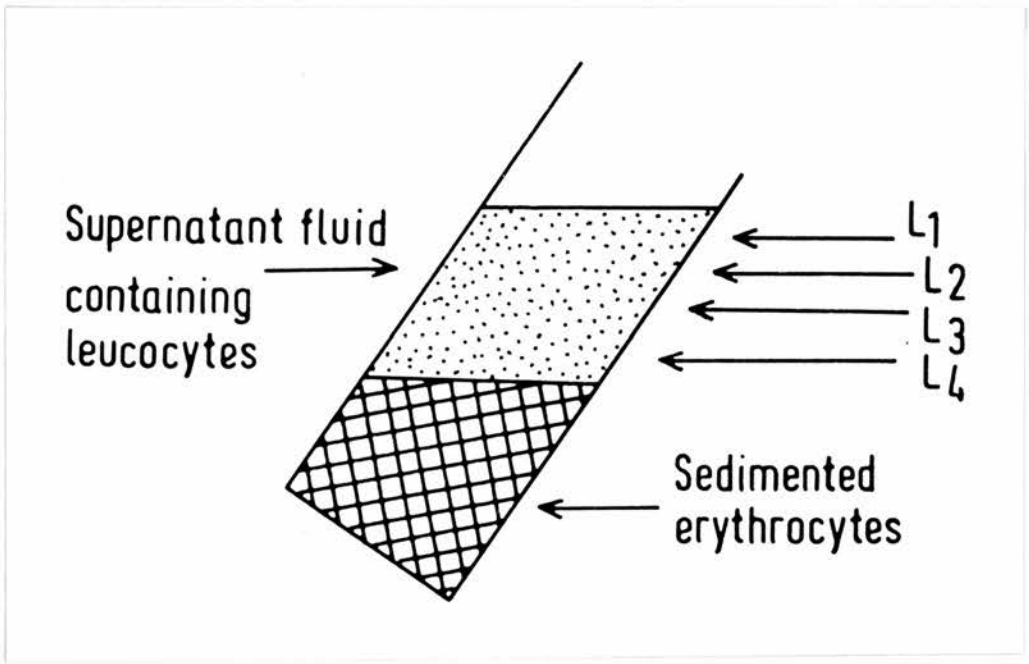


FIG. 3.4: Following incubation with NBT, neutrophils were sampled from 4 levels (L_1-L_4) of the supernatant fluid.

Results

Time of incubation

Table 3.1 shows the mean values for each of the three individuals tested for incubation for three time periods. The results are expressed as percentage of positive cells with the standard error of the mean shown.

TABLE 3.1: EFFECT OF INCUBATION TIME ON NBT REDUCTION (PERCENTAGE POSITIVE CELLS ± sem) BY PVB NEUTROPHILS

			Incubation Time (min)		
Subject	Sex	Age	20	30	40
MJK	M	28	1.3 ± 1.1	2.0 ± 1.0	1.7 ± 0.9
GHM	M	35	4.0 ± 1.2	2.5 ± 0.9	3.2 ± 1.6
LB	F	22	5.7 ± 1.7	4.0 ± 0.8	2.9 ± 1.4

From these few results, it was apparent that the test was functioning as anticipated in that the percentage of positive cells was low, with little variation between the values for the three incubation periods. For the sake of conformity, 30 min was chosen as the standard incubation time.

Incubation in air and water

The mean percentage positive cells from the test performed after 30 min incubation in air was 3.5% ± sem

0.5 and in water was 3.9% ± sem 0.6. Comparison of these means by Student's 't' test revealed no significant difference. However, in the light of Björkstén's (1974) observations, it was proposed to continue with water bath incubation. The temperature would also be less likely to fluctuate if, for example, the incubator door were to be opened during the course of an experiment.

Effect of storage on blood samples

The effects of storing the blood samples prior to testing are shown in Table 3.2. Although the percentage of positive cells fluctuated with time, slightly more for the blood stored at room temperature, this was not marked. Storage at 4^0C produced only slight variation, so that in accordance with accepted principles, blood samples were subsequently stored at 4^0C, and the NBT incubation sequence commenced within 4 hours of venepuncture.

TABLE 3.2: EFFECT OF STORAGE ON PVB NEUTROPHIL NBT REDUCTION (Results expressed as percentage positive cells)

Time of storage (h)		0	2	4	8	0	2	4	8
Temperature of storage	23^0C	3.1	6.1	2.9	5.8	2.1	1.4	4.9	6.1
	4^0C	3.1	4.6	2.9	3.4	2.1	1.9	3.2	4.2

GHM = 35 yr old male

MJK = 28 yr old male

Effect of sedimentation on NBT reduction

Table 3.3 shows the mean percentage of positive cells obtained from tests on three subjects. Cells were sampled from levels L_1 - L_4 as shown in Fig. 3.4.

TABLE 3.3: PERIPHERAL VENOUS BLOOD NEUTROPHIL NITROBLUE TETRAZOLIUM REDUCTION

			Mean Percentage of Positive Neutrophils at Four Levels			
Subject	Sex	Age	L_1	L_2	L_3	L_4
MJK	M	28	4.2	5.1	2.9	4.0
GHM	M	35	5.4	4.9	3.6	4.6
PG	F	21	2.1	1.8	2.6	3.1

No difference in reduction by neutrophils sampled from different levels was detected. Subjectively there was no difference in the numbers of neutrophils on the preparations from each level. As a point of note, eosinophilic granulocytes were prominent in levels L_1 and L_2.

Application of stimulants to NBT tests

From Table 3.4 it can be seen that in each case in which a stimulated test was performed, the neutrophils demonstrated an elevated response. Clearly the extent of the increased NBT reduction was variable, but application of Student's 't' test to the means of control

and stimulated results in each case showed the increase to be statistically significant ($p < 0.001$).

TABLE 3.4:

EFFECT OF MICROBIAL AND ENDOTOXIN STIMULANTS ON NITROBLUE TETRAZOLIUM REDUCTION TEST

	Percentage of Positive Cells ± sem	
Stimulant	Control	Stimulated value
Endotoxin	3.3 ± 1.3	25.6 ± 3.0
	7.2 ± 1.6	59.2 ± 3.4
	7.2 ± 1.6	37.3 ± 2.8
	0.2 ± 0.3	21.0 ± 2.2
	1.5 ± 1.3	21.3 ± 2.4
	1.8 ± 1.5	60.2 ± 9.1
	3.5 ± 1.6	24.3 ± 4.1
Staphylococcus aureus	4.2 ± 1.1	16.9 ± 1.6
	6.0 ± 1.4	38.0 ± 3.2
Enterococcus	4.6 ± 1.1	23.6 ± 2.4
Neisseria gonorrhoeae strain:		
6967	5.5 ± 1.5	23.0 ± 1.5
F62	8.0 ± 0.7	30.1 ± 0.9
M8131	7.1 ± 2.1	25.7 ± 4.2

Conclusions

Before the present study was undertaken there was a considerable amount of information available from the literature, both with regard to different methods for performing an NBT test, and some of the problems and technical factors that must be considered.

Having tried, unsuccessfully, to develop a glass-adherent assay which may have proved satisfactory given more time and effort, the method adopted was simple and easy to perform. The use of standard clinical equipment was also an advantage in that future development of the test for either clinical or purely laboratory research purposes would be facilitated. The limited experiments designed to verify published data on optimal test conditions supported the view that, if storage of blood is necessary, 4°C is preferable to room temperature but ideally blood samples should be processed within a few hours from collection. The functional capacity of neutrophil granulocytes is known to be impaired by freezing, but at least one group of workers has demonstrated a significant level of NBT reduction in neutrophils carefully reconstituted after freezing at -196°C in liquid nitrogen (Hill et al., 1978). This clearly has important implications for cell replacement therapy.

Sedimentation of neutrophils in this test system appeared to occur uniformly, although no specific quantitation was attempted. The development of positivity in cells and thus the accumulation of formazan did not appear to make the cells more dense for example and, as the results showed, sampling at any level would have been representative of overall activity.

The percentage of positive cells in the resting (or unstimulated) tests performed was low, and always less than 10%. The figures published from several groups of workers are shown in Table 3.5 and it can be seen that the results of the test described here were within the published range.

TABLE 3.5:
PUBLISHED RESULTS OF PERCENTAGE OF NBT-POSITIVE PERIPHERAL VENOUS BLOOD NEUTROPHILS

Authors	Mean and/or Range
Park et al., 1968	8.5
Andersen, 1971	0-9
Freeman and King, 1971; 1972b	1-11
Freeman and King, 1975	6.8 (3-11)
Gordon et al., 1973	6.1
Björkstén, 1974	0-13

The final test of validity was to ensure the ability of neutrophils in such a system to respond to a stimulant. When each of the stimulants was tested, invariably the response was increased as shown in Table 3.4. The degree of positivity was not, however, as high as in some other assays described (for example, Preisig and Hitzig, 1971).

Summary of the Peripheral Venous Blood Nitroblue Tetrazolium Reduction Test Method

Into a standard sequestrene tube are collected 2.5 ml peripheral venous blood to the marked line, and to this are added 2 ml saline-dextrose solution and 1 ml NBT solution containing 1 mg of NBT. The tube is incubated in a water bath at 37°C for 30 min, placed at 45° to the vertical. Following incubation, two 0.1 ml aliquots are centrifuged at 400 rpm for 5 min in a cytocentrifuge and the glass slide preparations stained with routine Romanowsky stain (e.g. Leishman Giemsa or Wright's). The positive cells exhibit the blue/black formazan staining, and a mean percentage positive count is made from five random field counts of 100 neutrophils per slide.

It was now possible to proceed to the development of a suitable NBT test for gingival crevicular neutrophils.

NITROBLUE TETRAZOLIUM REDUCTION TEST FOR GINGIVAL CREVICULAR NEUTROPHILS

Introduction

The principal objective was to apply an NBT test to crevicular neutrophils in order to determine their metabolic integrity. This had not been reported previous to this study.

As for the blood test, a number of experiments were undertaken to optimise conditions for NBT testing of crevicular cells. Once the system was shown to be satisfactory in the way described previously, it became possible to adapt the method to the exudate cells. It was soon apparent that some basic differences in the methodology would be essential, and these are described in the following section.

Materials and Methods

As for the blood samples, crevicular washings were taken from healthy adult donors and collected in the 2 ml plastic tubes as described in chapter 2. The crevicular rinsing fluid used, however, was the saline-dextrose solution (sds) and not phosphate buffered saline as

before. A fixed volume of crevicular cell suspension was then mixed with further sds and NBT solution, incubated and cytocentrifuge preparations made. The sds was clearly not required for sedimentation of erythrocytes but used for consistency. All microscopic counting and scoring of cells was performed as before at x 500 magnification with oil immersion.

Concentration of NBT

The final NBT concentration for the blood test was 0.18 mg/ml. To obtain similar conditions 0.05 ml of crevicular fluid suspension, 0.05 ml sds and 0.02 ml NBT solution were placed in a 2 ml plastic tube. The final concentration of NBT was therefore 0.17 mg/ml. The tube was incubated in a water bath for 30 min, cytocentrifuge preparations made, these stained with Leishman's stain and the scoring of positive cells done as before.

In view of the possible toxic effects of NBT on the crevicular cells, the experiment was repeated at double and half the concentration, i.e. a further 0.02 ml of NBT was substituted for 0.02 ml sds and 0.01 ml NBT solution was replaced by 0.01 ml sds respectively.

Incubation time

The morphology of cells in the cytocentrifuged preparations was less satisfactory than that for the untreated cells described in chapter 2. This may have been an unavoidable consequence of NBT toxicity but a further experiment was performed to investigate this on the basis that a shorter exposure to NBT might provide sufficient evidence of cellular activity with less cell disruption. Accordingly, the above experiment was repeated using NBT at a concentration of 0.08 mg/ml and 0.34 mg/ml, but with incubation times of 20 min and 30 min.

Influence of EDTA

Although it was not possible to create identical incubation conditions for both crevicular and blood neutrophils, one remaining factor that had to be examined was the absence of EDTA in the crevicular system. An attempt was made to investigate this in the following way. The EDTA in a sequestrene tube (4 mmol/l) is dissolved into 2.5 ml of blood and the volume of crevicular washing used in the above test was 0.05 ml. Thus, the crevicular volume was 0.2% that of the blood. Into an EDTA blood tube 2.5 ml sds were placed, shaken, and 0.02 ml used to substitute for 0.02 ml sds in the crevicular NBT test.* The original NBT concentration was 0.17 mg/ml and incubation was for 20 min.

*The final molar concentration of EDTA was therefore 0.67 mmol/l.

Effect of bacterial stimulation on crevicular neutrophil NBT reduction

In order to determine the extent to which crevicular cells may be stimulated further, the test was repeated in which a 0.05 ml suspension of Staphylococcus aureus at 1×10^8 organisms per ml harvested in log phase growth were substituted for the 0.05 ml sds normally incorporated. Incubation was at 37^0C for 20 min. A control experiment in which no additional bacteria were used was performed simultaneously.

Results

Concentration of NBT

Results of incubating cells with three concentrations of NBT are given in Table 3.6. The cells were collected from one healthy male subject aged 21 years.

TABLE 3.6: EFFECT OF NITROBLUE TETRAZOLIUM CONCENTRATION ON CREVICULAR NEUTROPHIL REDUCTION

NBT Concentration (mg/ml)	Percentage Positive Neutrophils (± sem)
0.085	20.7 ± 1.4
0.17	24.2 ± 1.2
0.34	< 10*

*This result is approximate because the cellular morphology was greatly disrupted so as to make scoring inaccurate.

The lowest concentration of NBT (0.085 mg/ml) clearly produced a reduction value close to that for the 0.17 mg/ml test, in neither of which was the extent of morphological disruption of the highest concentration seen.

Incubation time

The effects on NBT reduction of varying the incubation time with the three concentrations of NBT are shown in Table 3.7. The increase in reduction with the 0.17 mg/ml test was more marked, and the toxicity at 0.34 mg/ml confirmed. The percentage of positive cells produced at the two incubation periods was similar but subjective assessment showed that the shorter incubation preserved a much clearer morphology.

TABLE 3.7: EFFECT OF NITROBLUE TETRAZOLIUM CONCENTRATION ON REDUCTION VALUES FOR CREVICULAR NEUTROPHILS

NBT Concentration (mg/ml)	Mean Percentage Positive Cells (± sem) at 20 min and 30 min Incubation	
	20	30
0.085	20.4 ± 1.4	18.2 ± 1.9
0.17	25.8 ± 1.8	26.6 ± 2.3
0.34	**	**

** At this concentration cell disruption was considerable but more so in the cells incubated for 30 min.

Influence of EDTA

When EDTA was incorporated into the reaction, the percentage of positive cells was 27.1% ± 3.3% as compared to 23.8% ± 2.6% without EDTA. In this system there was therefore no evidence of a depression in the production or scoring of formazan-containing cells.

Effect of bacterial stimulation on crevicular neutrophil NBT reduction

In the control experiment NBT reduction by the crevicular neutrophils was 36.6% ± 2.0%, and in that incorporating Staph. aureus, 67.8% ± 4.2%. This difference was statistically significant when a Student's 't' test was applied to the means ($p < 0.001$).

Conclusions

As far as possible the crevicular NBT test was devised to be similar to that for the blood. In all these experiments the proportion of positive cells was far in excess of that for the blood studies on unstimulated cells. The implication is that the crevicular cells are therefore stimulated. Further stimulation of the cells demonstrated further NBT reduction, illustrating the metabolic potential still present.

The variation in preservation of cellular morphology of crevicular cells was noticeable. This had not been seen to such an extent in the peripheral blood tests although strongly staining blood neutrophils did show marked alteration in morphology. To improve the preservation of the crevicular cells, a standard incubation time of 20 min was shown to be acceptable.

A concentration of 0.17 mg/ml for NBT seemed appropriate, although it must be remembered that this is a volume-related calculation and not related to final cell number or concentration. To have achieved this for both blood and crevice would have made the test considerably more complicated.

Unlike the evidence regarding storage of blood prior to testing, the data presented in chapter 2 suggested that at 4°C satisfactory preservation of cells was obtained only up to 30 min from collection. In the case of crevicular cells 37°C was more suitable, but storage for up to 4 hours as for blood was not recommended.*

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* In order to confirm that the crevicular cells were vital throughout the incubation period, a simultaneous incubation as control was performed with one NBT test in which the NBT solution was replaced by sds. Samples were removed at 0, 10 and 20 min and the cells examined for viability by Trypan blue dye exclusion as detailed in chapter 2. The mean percentage viable cells based on five random counts at each time interval were 77.4% ± 2.9%, 78.7% ± 2.1%, and 81.7% ± 2.4% respectively. Outwith the influence of the NBT test itself therefore, the cell viability was unchanged during the 20 min incubation period.

Despite discussion in the literature on the relative merits and problems associated with different anticoagulants (Hellum and Solberg, 1973; Ward, 1974), incorporation of EDTA in the test system did not lower the activity of the cells at least in terms of formazan production. The reason for this is not clear except that it must be emphasised that all the experiments in this section were performed on cells from one subject. The objective was simply to develop a viable test which would subsequently be applied on a larger scale.

It was often observed that bacteria lying between cells on the slide preparations had apparently stained blue/black as with the formazan. It was assumed that either the Leishman stain had been concentrated in the bacterial cell walls or that the organisms had reduced the NBT to formazan. This point will be discussed further in a later section of this chapter.

Summary of the Crevicular Neutrophil Nitroblue Tetrazolium Reduction Test Method

Gingival crevicular neutrophils are collected with sds into a 2 ml plastic tube. A 0.05 ml aliquot is placed into a fresh 2 ml plastic tube to which are added 0.05 ml sds and 0.02 ml NBT solution (1 mg/ml). The tube is incubated in a water bath at 37^{0}C for 20 min after which 0.1 ml is removed and placed in a cytocentrifuge well. Cytocentrifugation is at 400 rpm for 5 min, after which standard Leishman-stained glass slide preparations are made and the percentage of formazan-containing neutrophils determined. Clearly it is advisable to perform the experiment in duplicate.

Having now developed satisfactory assays for NBT reduction by both peripheral blood and gingival crevicular neutrophils, a study was undertaken to investigate both in a group of volunteer subjects.

NITROBLUE TETRAZOLIUM REDUCTION BY PERIPHERAL BLOOD AND GINGIVAL CREVICULAR NEUTROPHILS IN 20 HEALTHY ADULT SUBJECTS

Introduction

In the development of an NBT test, it had been shown that circulating neutrophils exhibited a very low degree, and the exudate neutrophils a much higher degree of activation. Based on these limited data, it may be assumed that the former cells were giving a true resting value while the latter were demonstrating their stimulated nature, presumably in response to the bacteria in the gingival crevice. The purpose of this part of the study was to determine the extent of NBT reduction in blood and crevicular neutrophils sampled simultaneously from a group of systemically healthy subjects.

Materials and Methods

Ten male and ten female adult subjects were chosen for this study. All were systemically healthy, taking no drugs and had clinically healthy gingival tissues (Gingival Index < 0.02; Löe and Silness, 1963). The following routine was adopted for cell collection. From

each subject 5 ml PVB were taken and divided into two sequestrene tubes as previously described. From some subjects additional blood was taken for an estimation of total white cell count and differential, but this was not routine. A 0.05 ml sample of the blood was used for vitality testing by Trypan blue dye exclusion. Gingival crevicular fluid was then immediately collected into a 2 ml plastic tube. A total of 0.3-0.4 ml were collected in order to have sufficient cells for the subsequent tests. The NBT test was then conducted as previously described for blood and crevicular fluid, each in duplicate.

Having removed a total of 0.1 ml of the crevicular washings for the NBT test, two further 0.1 ml aliquots were spun onto glass slides on the cytocentrifuge in order to obtain a differential cell count. One was subsequently stained with Leishman's stain and the other for esterases as described in chapter 2. A final small sample was used to determine the vitality of the cells by Trypan blue dye exclusion. Much of this was accomplished during the incubation of the NBT samples. After 20 min, the crevicular cell incubation was terminated and the resultant samples spun onto glass, soon after which the procedure was repeated for the blood samples. This permitted more efficient use of the cytocentrifuge.

Results

Cell viability

All the leucocytes from the blood samples were vital as assayed by Trypan dye exclusion. The mean value for the crevicular cells for the 20 subjects was 78.04% ± sem 0.8% vital.

Differential cell counts of crevicular cells

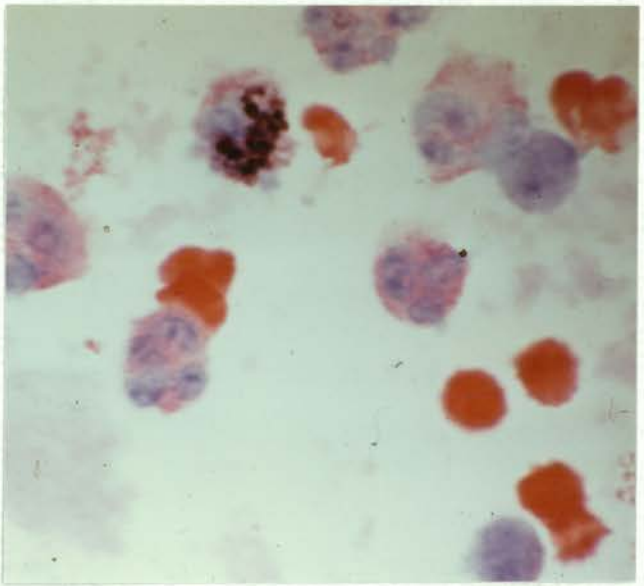
Based on morphological criteria, for the Leishman stained preparations the differential count was 95.8% ± 0.6% neutrophils, 2.4% ± 0.5% monocytes and 1.8% ± 0.3% lymphocytes.

With the esterase method the values were 98.0% ± 0.5% neutrophils, 1.6% ± 0.6% monocytes and 0.4% ± 0.2% lymphocytes.

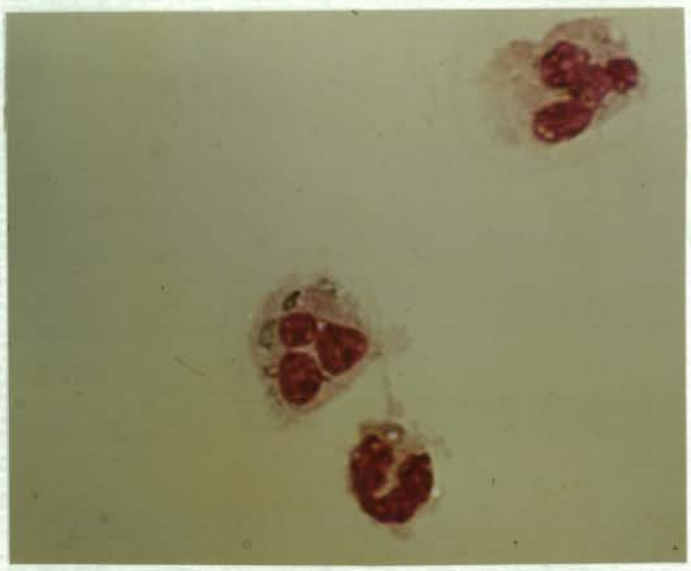
NBT reduction

Examples of NBT positive neutrophils are shown in Fig. 3.5a for blood and Fig. 3.5b for crevicular cells. The latter show weak staining but in Fig. 3.5c a much more strongly stained crevicular cell is seen with considerable disruption to structure.

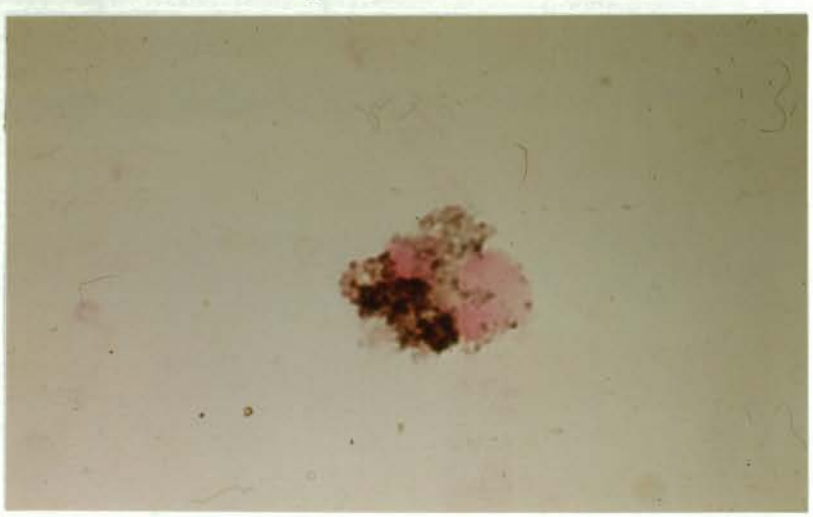
The mean values for percentage positive neutrophils in blood and crevicular fluid for each of the 20 subjects



a



b



c

FIG. 3.5:

Cytocentrifuge preparations of NBT positive neutrophils. Wright's stain. x 1200.

a) Peripheral blood cells

b) Crevicular neutrophils showing weak reaction

c) Disrupted crevicular neutrophil, showing strong positive staining.

are shown in Table 3.8. The means were calculated for each subject on the basis of ten random counts of 100 cells from the two slides. Clearly, for each group of cells there was a fairly wide range of mean values (0.8% to 13.8% positive for blood and 16.0% to 68.8% positive for crevicular cells). Comparison of the means for blood and crevicular cells by Student's 't' test indicated a significant difference ($t = 10.91$, $p < 0.001$). There was no significant difference for mean values of either blood or crevicular cells between the ten male and the ten female subjects ($p > 0.5$, NS). A distribution of the results for the blood and crevicular cell values was plotted, with 20% increments for the axis designating NBT positivity. Figure 3.6 shows this distribution to be heavily grouped at the lower values for the blood, whereas for the crevice the results are widely distributed over the higher range.

Further analysis was performed by plotting a linear regression of crevicular on blood values (Fig. 3.7), by calculating the slope and the 'best fit' lines for the male, female and total group data. The correlation coefficient for each was also determined and the method of analysis is outlined in Appendix 6.

The correlation coefficients, r, were as follows:

a) For the male group, $r = 0.52$. Degrees of freedom = 9, therefore not significant at 5% level.

TABLE 3.8:

MEAN PERCENTAGE NBT-POSITIVE CELLS FROM BLOOD AND CREVICULAR FLUID FROM 20 SUBJECTS

	Age (yrs)	Blood	Crevicular Fluid
Male subjects			
	35	1.3 ± 0.4	16.0 ± 1.2
	28	0.8 ± 0.2	24.2 ± 2.4
	45	2.1 ± 1.0	29.1 ± 1.3
	25	1.5 ± 0.4	42.6 ± 2.4
	22	13.4 ± 1.7	58.9 ± 2.3
	21	11.9 ± 0.7	54.4 ± 1.0
	21	2.6 ± 0.5	48.6 ± 3.5
	21	3.1 ± 0.6	61.4 ± 2.8
	24	4.8 ± 1.0	23.4 ± 1.5
	22	3.3 ± 1.2	57.9 ± 2.3
Means for 10 subjects	26.4	4.5 ± 1.4	41.7 ± 5.4
Female subjects			
	22	3.1 ± 0.5	27.6 ± 1.7
	18	7.1 ± 0.7	68.8 ± 1.4
	21	11.5 ± 1.0	46.0 ± 2.0
	21	4.8 ± 0.5	47.8 ± 2.4
	28	12.5 ± 1.2	37.1 ± 2.0
	23	6.8 ± 0.9	35.3 ± 2.3
	22	3.2 ± 1.0	44.7 ± 1.0
	27	5.1 ± 0.7	32.4 ± 2.1
	22	4.4 ± 1.0	43.7 ± 2.6
	21	9.3 ± 1.8	59.9 ± 2.5
Means for 10 subjects	22.5	6.8 ± 1.1	44.3 ± 4.0
Total mean for 20 subjects	24.5	5.6 ± 0.9	42.9 ± 3.3

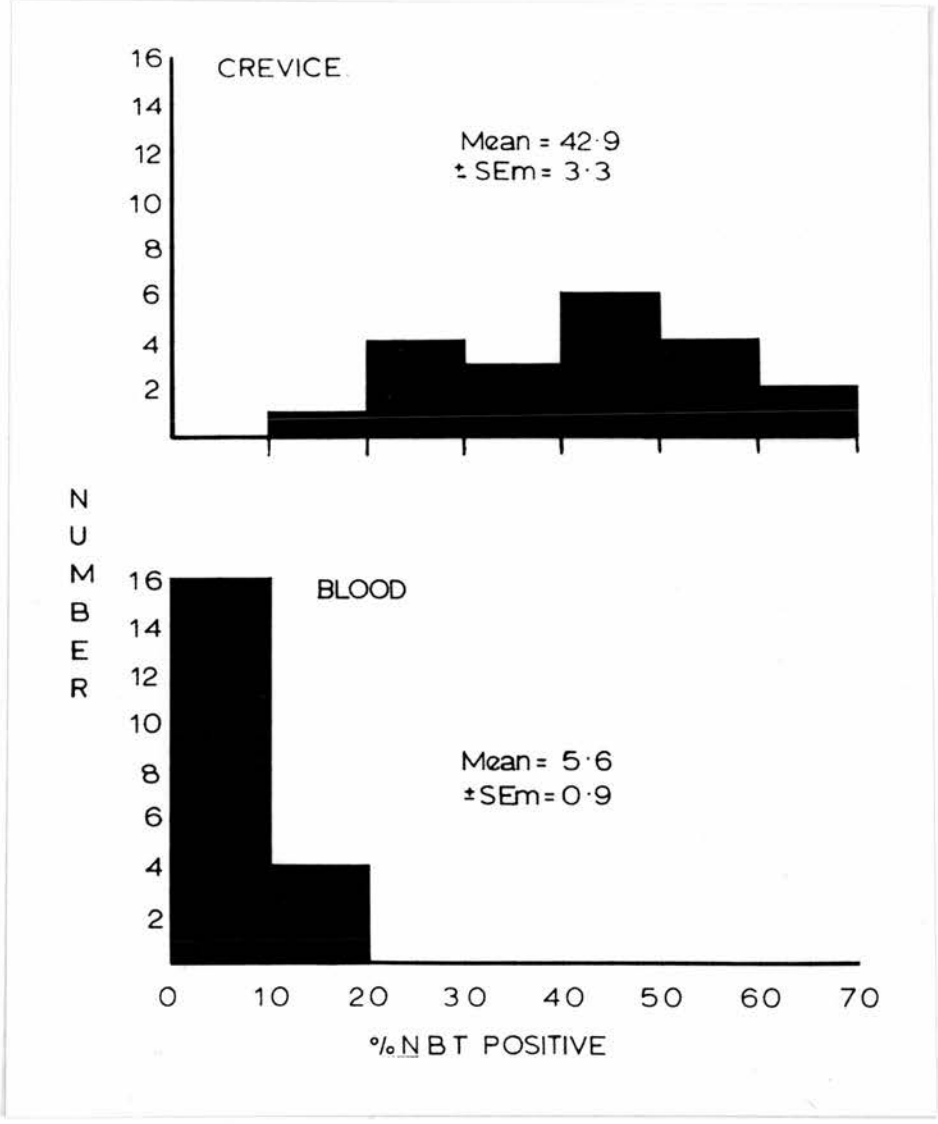


FIG. 3.6:

Distribution of peripheral blood and crevicular neutrophil NBT results.

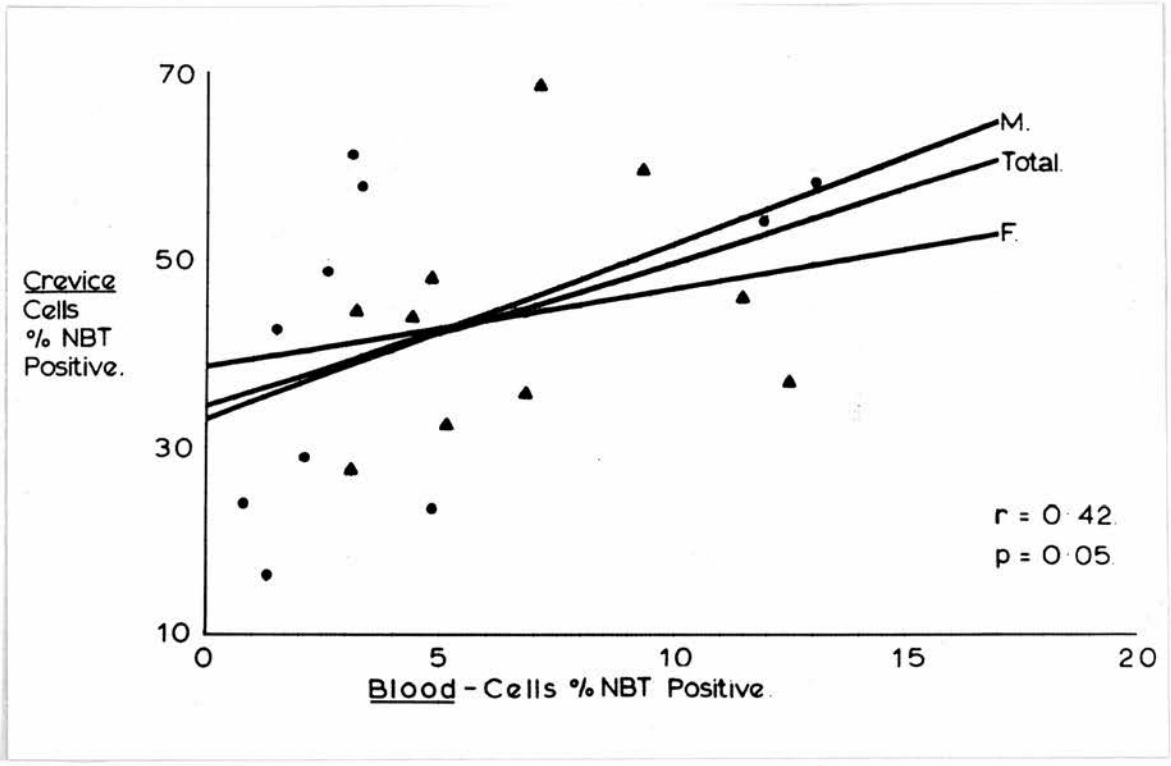


FIG. 3.7:

Linear regression of crevicular on peripheral blood neutrophil NBT values for 20 healthy subjects.

● - male

▲ - female

b) For the female group, r = 0.23. Falls far short of 5% level of significance.

c) For the total group, r = 0.42. Degrees of freedom = 18; therefore very close to significance at 5% level (at 5% level, p = 0.05, r = 0.44).

Thus, for the total group there was some indication that crevicular and blood neutrophil NBT reduction values were positively correlated.

Conclusions

The results from these experiments demonstrate that gingival crevicular neutrophils are stimulated cells in comparison to neutrophils sampled simultaneously from the peripheral blood. Even where no clinical inflammation is present, the gingival crevice contains bacteria, endotoxins and antigen-antibody complexes (Simon *et al.*, 1972; Wilton, 1977), which may result in membrane activation. Furthermore, in chapter 2 data were presented from subjects with tissues in a similar state of clinical health to those in this study, in whom *in vivo* phagocytosis could be demonstrated in approximately a quarter of the crevicular neutrophils. By direct inference the data also demonstrate the ability of these cells to respond to the stimulus present in this "*in vivo* stimulated NBT test".

The small positive correlation between the stimulation levels in blood and crevicular cells is of interest and may indicate a potential for microbicidal ability in a given individual. That is, using linear regression to predict a value for crevicular activity from a given blood value may have significance in predicting the outcome of long term confrontation between host and bacteria, and thus disease. At this stage such a hypothesis is mere speculation. However, systemically healthy individuals have only a small proportion of circulating neutrophils which show a positive response (Segal, 1974), and the results presented suggest that the gingival crevice may be one source of bacteria or bacterial products giving rise to that stimulation. It is well known that vigorous mastication or toothbrushing, particularly when gingivitis is accompanied by marked hyperaemia and oedema, will give rise to a transient bacteraemia. The consequences of this in immunodeficient patients are well known to haematologists. This may provide one explanation. It has been pointed out from a different perspective that, since neutrophils do not recirculate from the tissues and it is circulating cells which show an increased response in infection, somehow the signal must be transmitted back and produce the metabolic change (Harkness, 1981). In this paper it was further emphasised that the inflammatory process rather than the bacteria may be responsible.

The results of the vitality estimations and the differential leucocyte counts accord with those shown in chapter 2.

In summary, it has been shown that in terms of ability to reduce NBT to formazan and thus demonstrate an intact metabolic burst, the crevicular cells must be considered a functional population of phagocytes. One final technical point must be made here. All cells, whether vital or not, were included in the microscopic analysis when counting NBT positive cells, since presumably non-vital cells also take up the counterstain. Thus, corrected for a mean viability of 78%, the group mean NBT positivity would have been approximately 55% of potentially functional cells.

Footnote to this study:

Having established the clear difference in NBT reduction levels between neutrophils of blood and crevicular origin, a small investigation was performed to determine the values in age matched subjects with gingivitis. Four male and two female systemically healthy subjects were chosen. Each had a clinically evident gingivitis (G.I. > 1.5; Löe and Silness, 1963). The NBT tests were performed precisely as in the main study. For blood neutrophils the group mean was 9.6% ± 1.6% positive cells, and for crevicular neutrophils 44.2% ± 3.9%.

Although these limited results do not permit statistical comparison with the main study, the indication is that both blood and crevicular cells are in a similar metabolic state whether there is clinically obvious inflammation or not. Clearly, a larger study would be required to properly verify this point.

NITROBLUE TETRAZOLIUM REDUCTION BY WHOLE HUMAN SALIVA

Introduction

During the course of the previously described experiments on crevicular neutrophils, a chance observation revealed that at room temperature whole human saliva reduced NBT to its formazan over a comparable time period to that for the cell suspension. That is, a clearly visible blue colouration developed within 20-40 min of mixing the reagents.

Whole saliva is in fact a mixture of the secretions of the three main paired salivary glands, the minor mucosal salivary glands and the gingival crevicular fluid, and so contains many neutrophil leucocytes, most of which migrate from the gingival crevice and may be degenerating (Raeste, 1972c). Thus, one possible source of NBT reduction in saliva may be these cells. Saliva is a very complex fluid and varies in composition, and its reducing capacity although well known, has never been properly identified or localised. In a text book chapter of 75 pages on saliva, Jenkins (1978: 318) has discussed the oxidation-reduction activity in two very small paragraphs. He points out that the net reduction has been erroneously attributed to glucose.

Apart from the leucocytes, there are of course many agents in saliva which may influence the reducing capacity, e.g. glutathione, inorganic ions, ascorbic acid and other free enzymes (Tenovuo and Valtakoski, 1976). The involvement of bacteria in this context has been inconclusively discussed (Pincus, 1940; Eisenbrandt, 1943), but a significant correlation has been shown between the quantity of sediment and the oxidation-reduction potential, Eh (Nikiforuk, 1954). The sediment of whole saliva comprises approximately 86% epithelial cells, 13% leucocytes and 1% bacteria by volume (Klinkhamer, 1968b) which would suggest that any one of these could be the main source of NBT reductase activity.

This study was designed to identify if possible the agent or group of agents responsible for the marked reducing properties of human whole saliva. All experiments were performed on saliva collected without stimulation since the original observation had been made on such a sample. Saliva collected after stimulation differs in some significant respects (Jenkins, 1978: 284-359).

Materials and Methods

The approach adopted was one of identification by elimination; that is various components of whole saliva were investigated independently after the removal of other components or their activity. All tests were conducted in triplicate on 2 ml samples (unless otherwise stated) of whole saliva freshly collected without stimulation (by spitting into plastic test tubes) from systemically healthy adult volunteers exhibiting no clinical signs of active oral disease.

In each NBT test the 2 ml saliva sample was incubated in a plastic test tube with 0.3 ml NBT solution (Sigma standard NBT solution 1 mg/ml as previously described) in a water bath at 37°C for 10 min. This relatively short incubation period was always sufficient to allow for reduction of the NBT in a 'positive' experiment. When no colour change was visualised, the incubation was always allowed to continue for 30 min in the event of there being a slower reaction.

In studying the involvement of bacteria, following each particular isolation procedure and prior to NBT testing, single standard loopful samples of treated saliva were inoculated onto blood agar plates and cultured aerobically overnight at 37°C. Subsequent bacterial growth was assessed subjectively as positive (heavy or

light growth) or negative. The assessment of NBT reduction was also as positive (strong or weak) or negative.

Physical methods of assessment

The objective was to distinguish activity between the sediment and supernatant fraction of whole saliva.

a) Centrifugation and isolation of the salivary pellet

Saliva samples (2 ml) were centrifuged at 2,500 *g* for 30 min after which the pellet was resuspended in 2 ml phosphate buffered saline (PBS). The resuspended pellet and the original supernatant fluid were each tested for NBT reducing activity.

The three main constituents of the pellet i.e. epithelial cells, leucocytes and bacteria, were then examined individually. From the buccal mucosa,scrapings were made to obtain epithelial cells, which were suspended in PBS. These cells were then washed twice by centrifugation and resuspension in PBS, after which the test was performed along with the standard bacteriological plating. The leucocyte fraction was studied indirectly by shaking saliva samples in an excess of distilled water in order to lyse the leucocytes. The samples were then centrifuged and the pellet resuspended in PBS and tested as before.

The contribution of the salivary bacteria will be discussed more fully below.

b) Filtration,dialysis and freezing

Samples of saliva were filtered through a millipore filter (Millipore UK, Ltd., Harrow, Middlesex), size 0.25 μm, and the filtrate tested for NBT reducing capacity and bacterial contamination. Dialysis of whole saliva against normal sodium chloride was performed for 24 hours at 4°C and the dialysate tested. Finally, samples were frozen for three weeks at -40°C, rapidly thawed and tested without further manipulation.

Bacteriological methods of assessment

The contribution of bacteria was examined by treating saliva samples in three ways:

a) Pasteurisation
b) Incubation with aqueous chlorhexidine gluconate
c) Incubation with antimicrobial drugs

a) Pasteurisation: Samples of saliva were collected and divided into two. One sample was allowed to stand at room temperature as control and the other was pasteurised by incubation for 45 min at 60°C. NBT testing and bacteriological plating were undertaken as before.

b) Incubation with aqueous chlorhexidine gluconate: As for pasteurisation, 2 ml samples of whole saliva were

incubated for 1 hour with 1 ml of 2% w/v aqueous chlorhexidine gluconate and tested.

c) Incubation with antimicrobial drugs: Samples of saliva were incubated for 3 hours at 37^{0}C with benzyl penicillin at a final concentration of 100 mg/ml and nalidixic acid at a final concentration of 15 μg/ml. NBT testing and assessment of bacterial growth were conducted as before.

Further investigation of the effect of penicillin was undertaken by incubating with saliva at final concentrations of 0, 1, 10, 50, 100 and 250 mg/ml prior to testing. Following incubation with NBT, a quantitative reduction assay was then performed and the optical density of each sample determined. In order to do this, a calibration curve had to be drawn to determine the optimum wavelength at which to monitor the reactions. A Pye Unicam SP600 Series 2 spectrophotometer was used (Pye Instruments, Cambridge, UK) and, as a blank with which to perform the calibration, 3 ml NBT solution (1 mg/ml) in the glass phial. The calibration of optical density against wavelength is shown in Fig. 3.8. The optimum wavelength so determined was 540 nm.

The experiment was conducted as follows. Saliva samples were incubated with increasing concentrations of benzyl penicillin up to 250 mg/ml as stated above for 3 hours at 37^{0}C. Each sample was then thoroughly mixed,

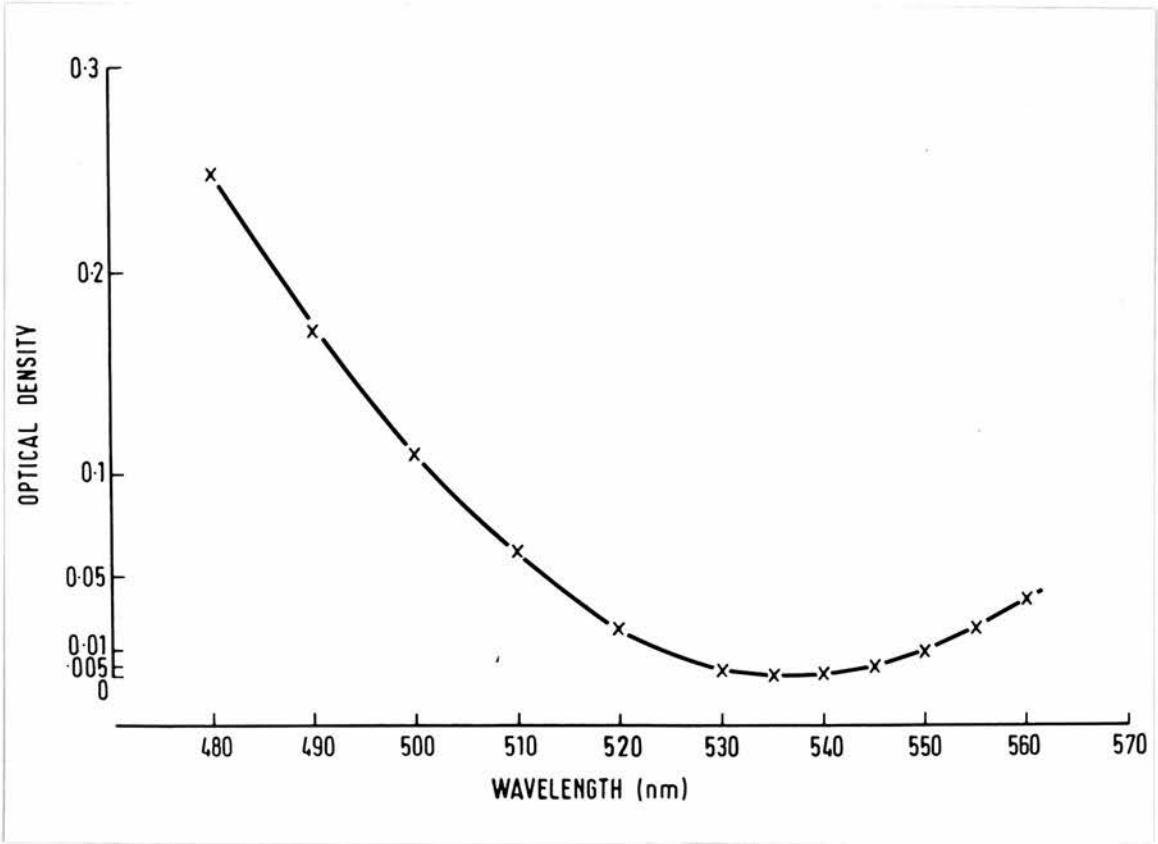


FIG. 3.8:

Absorption spectrum for the spectrophotometric assay of formazan produced from NBT reduction by saliva samples.

It was assumed that there was a linear relationship between absorption at 540 nm and the volume of saliva.

2 ml removed and reincubated with 0.3 ml NBT solution for 30 min at 37°C. As before, samples were plated on blood agar after incubation with penicillin to determine the extent of residual bacterial growth. After incubation with NBT the optical density of each sample was assayed. It was soon apparent that, even where the higher concentrations of penicillin had been used, the intensity of the blue colouration was too great to permit readings to be taken. By experimentation it was found that a standard dilution of the sample six times with PBS permitted readings to be made at all concentrations of penicillin.

NBT reduction by salivary bacteria

From the large number of saliva samples incubated on blood agar, it became possible to identify morphologically those bacterial species which were quantitatively prominent or distinctive. These were subcultured and identified by their appearance on blood agar and by Gram staining. They were then grown on blood agar aerobically, and from overnight cultures, harvested and suspended in PBS at a concentration of 1×10^9 bacteria per ml. These suspensions were then incubated for 30 min at 37°C with NBT solution. As before, 0.3 ml NBT and 2 ml of bacterial suspension were the volumes used. Subsequent NBT reduction, if present, was monitored subjectively as in the initial experiments.

Additional tests

Pure parotid saliva was collected using a Carlsen-Crittenden cannula which comprises two circular concentric chambers to each of which is attached plastic tubing and a disposable syringe. The inner chamber was placed over the opening of the parotid duct and suction applied to the outer chamber, so forming a seal with the mucosa of the cheek. The syringe attached to the inner chamber was then used to withdraw the parotid secretion from the duct. Parotid saliva was thus collected and, being lesser in volume than whole saliva, 0.2 ml samples were incubated with 0.03 ml NBT solution.

The relevance of salivary glucose was determined by incubating whole saliva samples of 1 ml with 0.1 ml glucose oxidase, thus removing the glucose before incubation with NBT.

Results

In cases where the NBT reduction was positive, the intense blue colouration was always produced within 10 min incubation period. In no case was a slower reaction seen. The concentration of NBT used (0.3 mg in a total of 2.3 ml = 0.13 mg/ml) thus gave a very clear positive result. Examples of positive and negative reactions are

shown in Fig. 3.9.

All the results and the effects of physical treatment are summarised in Table 3.9.

The results clearly showed that the NBT reducing activity of the whole saliva samples was entirely located in the sediment fraction. The epithelial cells and leucocytes in the saliva were insignificant in that the former produced no reduction and elimination of the leucocytes produced no diminution in the intensity of the reaction.

The process of elimination showed that all significant reducing activity was located in the bacterial fraction, and this was subsequently borne out both by direct experimental evidence and indirectly from the bacterial cultures.

Pasteurisation and incubation with 2% chlorhexidine gluconate totally abolished subsequent NBT reduction, and incubation with benzyl penicillin at a concentration of 100 mg/ml subsequently produced a mild positive reaction. This, and the fact that nalidixic acid did not affect the reaction, led to the more detailed experiment with a range of penicillin concentrations. The result clearly demonstrated that increasing the concentration of penicillin led to a decrease in subsequent NBT reduction, and these data are presented graphically in Fig. 3.10. The intensity of the positive reaction was again emphasised by

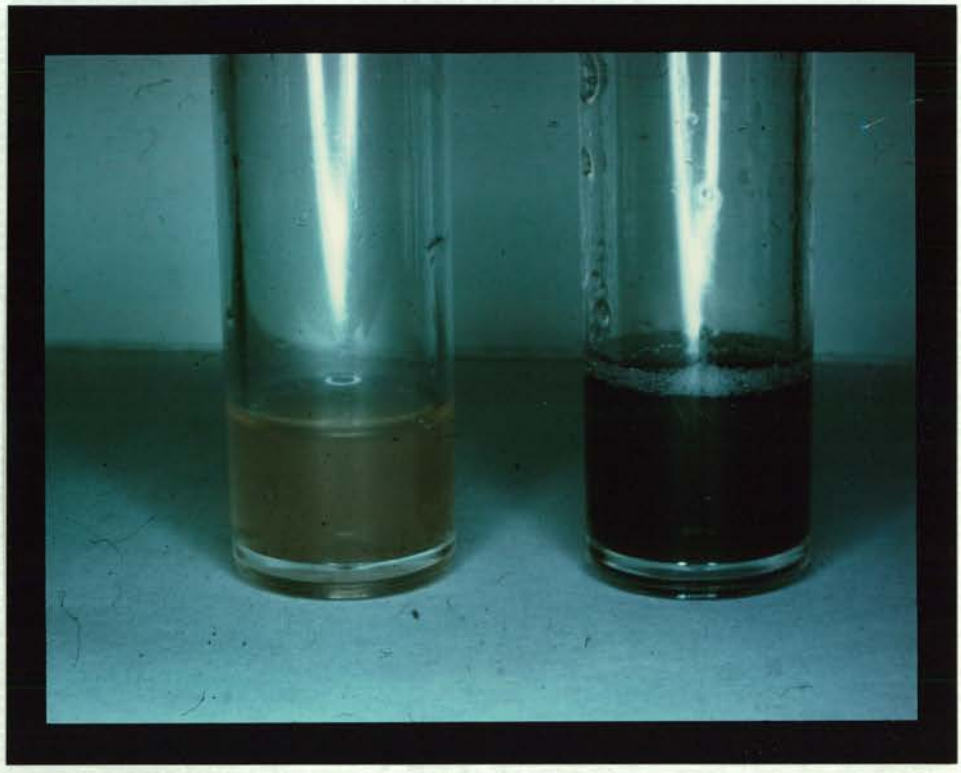


FIG. 3.9:

NBT reduction by human saliva. The strongly positive reaction is clearly seen in the tube on the right, while that on the left is negative.

TABLE 3.9:

EFFECT OF VARIOUS PHYSICAL AND BACTERIOLOGICAL PROCEDURES ON THE NITROBLUE TETRAZOLIUM REDUCING CAPABILITY OF HUMAN SALIVA AND CONCOMITANT AEROBIC CULTURE

Procedure	NBT test	Culture
Untreated saliva	++	++
Centrifugation - supernatant	-	-
- pellet	++	++
Pellet - epithelial cells	-	-
- after lysis of leucocytes	++	++
Filtration	-	-
Dialysate	++	++
Freezing	+	+
Pasteurisation	-	-
Chlorhexidine gluconate	-	-
Benzyl penicillin	-	-
Nalidixic acid	++	++
Parotid saliva	-	-
After glucose elimination	++	++

++ = strong positive reaction

\+ = mild positive reaction

\- = negative reaction

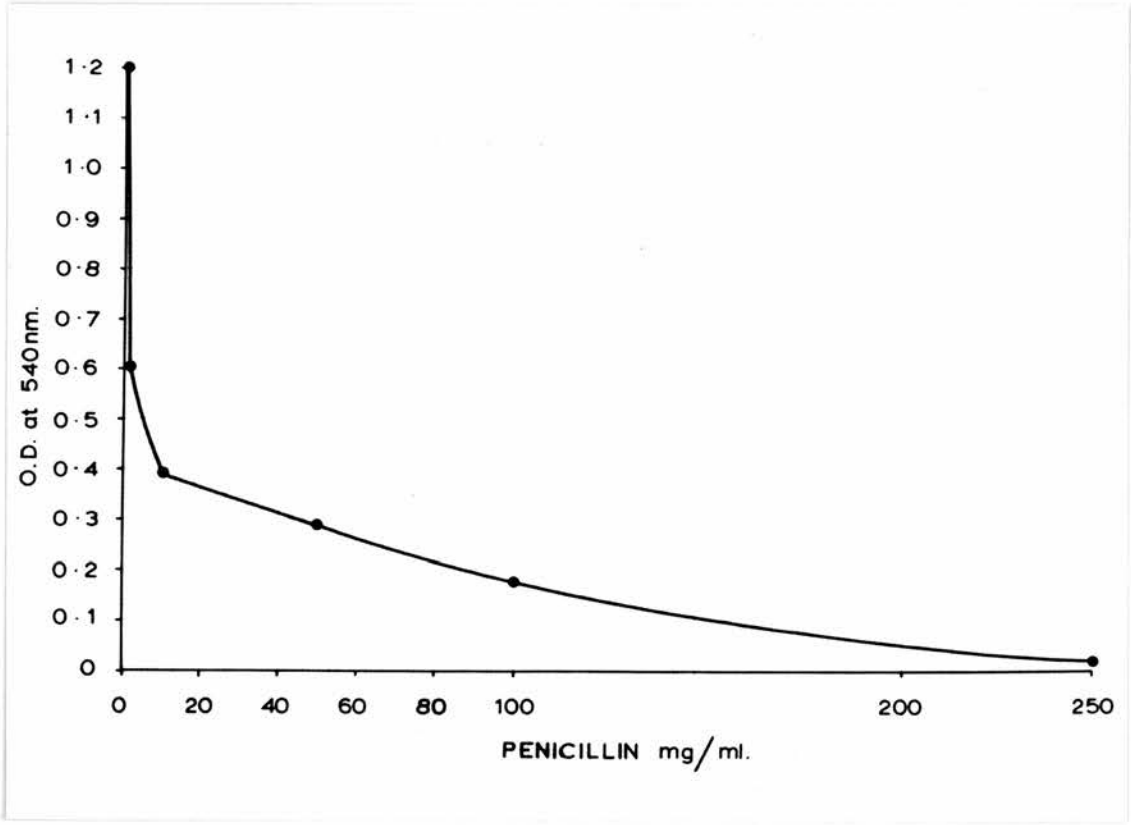


FIG. 3.10:

Spectrophotometric assay of formazan produced from NBT reduction by saliva samples pre-incubated with benzyl penicillin. Inhibition of reduction increases with increased concentration of penicillin.

the necessity to dilute the samples six times in order to permit transmission of light. The evidence for bacterial involvement was reinforced by the concomitant decrease in subsequent bacterial growth with increasing antibiotic concentrations, and examples of the growth plates are shown in Fig. 3.11.

As shown in Table 3.9, parotid saliva had no reducing activity, and the removal of glucose did not diminish the reaction intensity.

The above results strongly suggest that salivary bacteria were responsible for the very intense and rapid reduction of NBT to formazan. The species identified as being most prominent numerically were viridans streptococci, Branhamella catarrhalis and members of the genus Micrococcacea. Only the latter gave a convincing positive result with NBT and the reaction was extremely rapid and the colour intense.

Conclusions

This investigation into an area which had previously been little examined and largely abandoned, stemmed from a chance observation. It seemed logical to pursue the identification of the reducing activity in whole saliva for two reasons. Firstly, it extended experience of

FIG. 3.11:

Indication of the reduction in salivary bacterial flora as a result of pre-incubation of saliva samples with benzyl penicillin. Blood agar plates incubated aerobically overnight at 37^{0}C. From upper left to lower right, the penicillin concentrations were 0, 1, 10, 50, 100 and 250 mg/ml.

using nitroblue tetrazolium experimentally and led to its use in a quantitative spectrophotometric assay. Secondly, and more importantly, saliva contains neutrophil leucocytes at a concentration of approximately 200 to 400 cells per ml (Calonius, 1958) and it was supposed that these might contribute significantly to the observed reduction of NBT, so indicating their metabolic viability. This latter assumption was not upheld, but in the process of tracing the activity to bacteria, some experience was gained in basic laboratory microbiological techniques. In all cases where bacteria were reduced in number or eliminated, the subsequent NBT reduction showed a parallel decline. One considerable advantage was the very clear result given by the experimental conditions employed.

The initial results, using antimicrobial methods, i.e. pasteurisation and the antiseptic chlorhexidine gluconate, were conclusive except that it was possible for the physical conditions of the former and the very powerful cationic character of the latter to have produced denaturing of some active reducing agent in the whole saliva. Thus, the study was extended and the results reinforced by the use of more specific antimicrobial drugs. Nalidixic acid is bactericidal to Gram negative anaerobic organisms at the concentration used (Deitz et al., 1963). The experimental approach could not otherwise eliminate the involvement of these species, but the lack of

inhibition in reduction to a visible extent suggested that the aerobic or facultatively aerobic Gram positive bacteria were responsible. This was strongly supported by the convincing dose response seen with penicillin which had a wide spectrum of activity against the species grown on the overnight cultures. Presumptive identification of the principal bacteria as Micrococcacea was based on the colony appearance, colour (yellow shiny colonies) and Gram positive nature. This genus certainly possesses NADH and metabolises glucose via the HMPS (Buchanan and Gibbons, 1974), so that the potential for electron transfer to NBT exists. It must be emphasised that the bacteriological methods employed were in some ways imprecise. For example, in plating the bacteria on blood agar, a single loopful of saliva was used in each case. Although the same loop was used throughout, it is unlikely that the original inoculum size was identical in each case. More precise identification of bacterial species was not attempted. This was felt to be beyond the scope of the principle upon which this particular study had been undertaken. The component of whole saliva responsible for the reducing activity as shown by NBT reduction had been isolated and more detailed bacteriological identification would have necessitated the use of chemical techniques and considerable expertise.

The experimental approach did not totally eliminate a contribution by glucose or the leucocytes, but simply demonstrated that neither was responsible in a major capacity. Having developed a spectrophotometric assay for NBT reduction, careful manipulation may have enabled any effects of these two agents to have been revealed.

Finally, there may be an obvious explanation for the inconclusive studies undertaken 30 to 40 years ago, which attempted to define the reducing activity in saliva (Pincus, 1940; Eisenbrandt, 1943; Nikiforuk, 1954). The means of separating the active constituents of the sedimented pellet were largely unavailable, and although some early antibiotic drugs would have been in clinical use, it is possible that their application as laboratory tools had yet to emerge. The identification of reducing activity in saliva as shown in this study was made more convincing by the use of benzyl penicillin.

DISCUSSION

The ingestion of bacteria and their subsequent destruction is regarded as the principal function of neutrophil phagocytes and the mechanisms by which these processes occur have received close attention, particularly over the past 20 years. The reduction of oxygen to active species is thought to play a key role in bactericidal events, and the generation of these species, so closely linked with the supply of pyridine nucleotides and glycolysis has received particular attention.

Considerable progress has been made through a fuller understanding of membrane biology (Elsbach, 1977; Henson, 1980). The nature of the stimulus, not surprisingly, may have a crucial role in determining the type of radicals, the relative proportion of these radicals and perhaps most importantly for how long they are produced (Dahinden et al., 1983). The nature of the NADPH oxidase has been the focus of collaborative studies worldwide, and has largely been elucidated.

When the functional pathways in host defence mechanisms are identified, they can be clinically investigated in patients. The NBT test was seen 10 to 15 years ago as a simple method by which to determine the integrity of the neutrophil metabolic burst, and possibly to establish the presence of bacterial infection. With

regard to the former, a well performed NBT test remains part of the diagnostic panel of tests crucial to the diagnosis of chronic granulomatous disease, in which the respiratory burst is absent (Baehner and Nathan, 1967). Unfortunately, however, due to the many reports of false positive and, more importantly in this context, false negative results being obtained, its use in the diagnosis of infection has never gained widespread support. It remains for individual clinical laboratories to use their own tests in which confidence is gained by experience.

The reduction of NBT by phagocytes has retained respectability in the field of applied research. It is often used in conjunction with other measurements of metabolic stimulation in neutrophils and, for example, has been shown to correlate with oxygen uptake more than with phagocytosis (Torres et al., 1979). Stavridis et al. (1981) have used a cross-sectional study to show that, throughout life, there is a parallel fluctuation in neutrophil NBT activity and alkaline phosphatase, and suggested from their data that both are under some degree of hormonal control.

The mechanism of reduction remains an issue of discussion but is either by electron transfer from the superoxide anion or from direct reduction by NADPH, or perhaps both. Baehner et al. (1975) have argued that, while solubilised fractions of neutrophils, including

those from patients with CGD, will allow NBT reduction by NADPH, in whole cells the generation of the superoxide anion is essential. In whole cells, no reduction occurs under anaerobic conditions. In principle, either mechanism relates to the metabolic burst and the superoxide involvement also implicates bactericidal pathways, thus supporting the relevance of NBT reduction to assays of neutrophil function.

The semi-quantitative histochemical tests, originally devised by Park et al. (1968) and Gifford and Malawista (1970) have received many modifications over the years and are easily performed. The quantitative methods based on Baehner and Nathan (1968) are probably more precise, but are technically more involved and obscure one interesting feature of NBT reduction by resting or stimulated neutrophils. That is, neutrophils that have been incubated with NBT show a proportion of cells on a histochemical preparation that have reduced the dye and a proportion that for some reason have not. Moreover, of these positive cells (which are grouped together when recording a test result), some are weakly positive and some so strongly positive that the cell is totally disrupted (see Fig. 3.5). It may be important to take account of this.

Further technical points must be considered. Balogh and Cohen (1961) observed fine granules of formazan on or

in erythrocytes and this was occasionally seen in the blood preparations made in the studies described in this chapter. This in no way interfered with the clarity of reading the test, but might be of relevance to a quantitative assay. At the outset, in developing the blood NBT test an advantage was seen in using standard clinical equipment and this proved to be satisfactory. However, it may be argued that the method is extravagant with the use of blood, in that far more cells are collected from the patient than are actually required. A proportional reduction in the volumes of all reagents could be employed, for example by using paediatric-sized collecting tubes.

The studies described have concerned the development of a peripheral blood NBT test and subsequently a test for gingival crevicular cells. The results of the former test were consistent with those of other workers, both for resting cells and cells stimulated with a range of agents. This test method is therefore considered suitable for application in the areas described above. The crevicular neutrophils were shown to be metabolically stimulated, presumably by the bacterial and/or inflammatory components of the gingival crevice. This was an important finding because previously only limited studies of phagocytosis and killing of Candida albicans had been conducted on these cells (Wilton et al., 1977a). Subsequently, one

other group of workers has confirmed that crevicular neutrophils are able to reduce NBT and are highly stimulated cells (Charon et al., 1982). These authors, however, were unable to make statistical deductions about the relationship between blood and crevicular neutrophils which was just possible in the present study by linear regression analysis. The small positive correlation demonstrated between the NBT reduction levels in the two cell groups was intriguing both from the point of view of a stimulus feeding back to the circulation and also by opening the question of whether a predictive test of crevicular cell function is possible from analysis of PVB activity. In a recently published study of human exudate (skin window) neutrophil NBT reduction, the resting rate of activity was significantly higher than that from blood neutrophils. There was no difference when both cell types were phagocytosing paraffin oil emulsion (Wandall, 1982). No comparable experiment to the latter was performed in this study but it would be of interest to compare stimulated as well as unstimulated blood neutrophil values to those for crevicular cells and to determine the maximum potential activity for the crevicular cells.

The limited data presented for patients with gingivitis suggested that both blood and crevicular neutrophils are in a similar state of activity as when oral tissues are healthy. Further studies would be required to confirm this but the similarity is in contrast to a study published

by Kusunoki (1977). He showed that peripheral blood neutrophils from patients with periodontitis had depressed levels of NBT reduction which returned to normal following clinical treatment. However, these values were 12.5% positive cells as opposed to over 25% for normal subjects which are very high for an unstimulated test. There was no phagocytic defect but the decrease in reduction by periodontitis neutrophils was attributed to some serum blocking factor. This again would be an interesting finding to pursue. He did not test crevicular cell function. In their series of patients with bacterial infection, Park et al. (1968) demonstrated slightly raised peripheral blood neutrophil NBT reduction including a patient with an acute gingival abscess.

Beyond any intrinsic merit in the study of salivary reduction of NBT, there was the possibility that the results could have a bearing on an understanding of host defences in the oral cavity. Thus, although it has been demonstrated that salivary neutrophils have a reduced phagocytic capacity as compared to crevicular cells (Scully, 1982), they may be sufficiently intact to reduce NBT. The experimental evidence did not uphold this supposition. However, one important point has emerged. The dye reduction by bacteria was extremely powerful so that if bacteria are used in a 'stimulated test', and they are themselves capable of reducing NBT,

then with an untrained eye, a false positive result may be reported. A similar result was reported for neotetrazolium chloride reduction by rat peritoneal exudate cells phagocytosing brucella 30 years ago (Shaffer et al., 1953).

Two final points must be made about the results for the crevicular neutrophil study. A practical advantage would be gained from being able to study aspects of cell function in a reliable manner from a small number of cells collected atraumatically by a non-invasive technique. Secondly, having established indirectly the integrity of the metabolic burst in a significant proportion of crevicular cells, the next step was to examine more closely a related but distinctly bactericidal function, that of myeloperoxidase.

CHAPTER 4

MYELOPEROXIDASE IN GINGIVAL CREVICULAR NEUTROPHILS

INTRODUCTION

The previous chapter considered the respiratory burst which, following activation, produces a number of oxygen radicals and ionic species. These are thought to be central to the oxygen-dependent microbicidal systems of neutrophils. Of particular interest are the oxidation reactions of hydrogen peroxide (H_2O_2), catalysed by the neutrophil peroxidase now known as myeloperoxidase (MPO)(Hydrogen peroxide = halide = oxidoreductase; E.C.1.11.1.7).

Historical Aspects - Lactoperoxidase and Myeloperoxidase

A peroxidase enzyme is not in itself bactericidal but is so indirectly by virtue of its catalytic properties in converting a substrate from a weak to a strong anti-microbial agent (Klebanoff and Clark, 1978: 410). Peroxidases are generally haem proteins containing variable amounts of iron and are extremely widespread in both animal and plant tissues. Over 50 years ago enhancement by peroxidase of the germicidal properties of phenol and cresols was noted (Kojima, 1931). The first studies of the mammalian enzyme were conducted on milk and saliva, i.e. secretions, even earlier. In 1924, Hanssen suggested that the antimicrobial properties of bovine

milk may be related to the content of "oxydases" or "peroxydases". In fact, the majority of studies which followed were conducted on milk or saliva and indeed many workers continue to investigate the bactericidal properties of these secretions. Through the 1950s and 1960s several studies were published establishing the antibacterial properties of the milk peroxidase termed lactoperoxidase (LPO) (Jago and Morrison, 1962). It was also shown that a secretory non-enzymatic co-factor was required, later to be identified as the thiocyanate ion (Reiter et al., 1964). Since catalase strongly inhibited the oxidation reaction, hydrogen peroxide was suggested as a component (Jago and Morrison, 1962). It was not incorporated as a reactant in the early experiments, but fortuitously many of the test organisms used were hydrogen peroxide producers, such as streptococci or lactobacilli.

Similarly, human salivary peroxidase was isolated (Klebanoff and Luebke, 1965), and shown to be almost identical to the enzyme from bovine milk (Morrison et al., 1965). This enzyme is also inhibited by catalase as shown with lactobacilli, which are hydrogen peroxide producing (Klebanoff and Luebke, 1965). Provided a source of hydrogen peroxide is included (which may be appropriate microorganisms), non-hydrogen peroxide generating organisms are also inhibited (Klebanoff et al., 1966). The salivary and milk antibacterial systems were thus seen as comprising peroxidase and thiocyanate

contributed by glandular secretion, and hydrogen peroxide contributed primarily by organisms. In human milk, however, the peroxidase has recently been shown to be derived from the leucocytes and is thus myeloperoxidase, with distinctly different separation characteristics (Moldoveanu et al., 1982). Lactoperoxidase is a single unit polypeptide chain with a molecular weight of approximately 78,000 daltons (Rombauts et al., 1967), whereas human peripheral blood neutrophil MPO comprises two sub-units and has a molecular weight of 120-140,000 daltons (Matheson et al., 1981). In whole human saliva analysis has shown the peroxidase (LPO) to be heterogeneous, with a mean molecular weight of 80-100,000 daltons (Slowey et al., 1968), but as stated above is very similar to bovine LPO in antigenic cross-reactivity. Saliva contains peroxidases with separate origins, i.e. bacteria, leucocytes and a glandular secretion, which are chromatographically distinct (Mäkinen and Tenovuo, 1976). In inflammatory exudates, including gingival crevicular fluid, the peroxidases at least in part are likely to be derived from cellular disintegration (Tenovuo et al., 1978).

Both LPO and MPO are able to catalyse thiocyanate-mediated reactions, but only MPO will catalyse the chloride ion oxidations (Morrison and Schonbaum, 1976). The oxidation of the thiocyanate ion (SCN^-) by hydrogen peroxide produces the hypothiocyanite ion ($OSCN^-$) at

neutral pH (Hoogendoorn et al., 1977) or hypothiocyanous acid (HOSCN) at a lower pH (Thomas, 1981):

$$H_2O_2 + SCN^- \longrightarrow H_2O + OSCN^-$$

or

$$H^+ + OSCN^- \rightleftharpoons HOSCN$$

The concentration of hypothiocyanite ion in human saliva varies with stimulation, being higher in resting saliva (Tenovuo, Pruitt et al., 1982), but hypothiocyanous acid may be the more bactericidal of the products, having a greater ability to penetrate bacterial cell membranes (Thomas, 1981). The bacterial damage is affected by the subsequent oxidation of protein sulfhydryl groups (Thomas and Aune, 1978a).

The salivary and milk peroxidase-mediated antimicrobial systems have been widely investigated for their ability to control a range of microorganisms. Of particular interest are potential pathogens. For example, efficacy has been demonstrated against Salmonella typhimurium (Purdy et al., 1983), Pseudomonas species (Bjorck et al., 1975), Escherichia coli (Thomas and Aune, 1978b; Stephens et al., 1979), and of particular interest in dental caries, against Streptococcus mutans (Tenovuo, Moldoveanu et al., 1982; Thomas et al., 1983). Tenovuo, Moldoveanu et al. (1982) have suggested that

secretory IgA in saliva may enhance the bactericidal properties of LPO. A positive correlation has been demonstrated between the state of health in the oral cavity (with respect to dental caries) and the concentration of hypothiocyanite in salivary sediment (Tenovuo and Anttonen, 1980). There is also accumulated evidence, both in animal models (Stephens et al., 1979) and humans (Cockle and Harkness, 1978; 1983), for one of the control mechanisms for peroxidase secretion being hormonal, specifically oestrogenic.

MPO is also able to catalyse the thiocyanate reaction (Klebanoff and Luebke, 1965) but in 1967 it was shown that the halide ions (iodide, bromide and chloride) could be successfully substituted for thiocyanate in the MPO-mediated system (Klebanoff, 1967). Many subsequent studies have confirmed the efficacy of this system against a range of microorganisms and viruses (Klebanoff and Clark, 1978: 412-413). It was simultaneously reported that the peroxidase-hydrogen peroxide bactericidal system of leucocytes (guinea pigs) could be isolated from a granule fraction of the cells (McRipley and Sbarra, 1967b).

Myeloperoxidase - Development and Properties

Agner (1941) first described neutrophil peroxidase as verdoperoxidase because the purified enzyme was green, but since this also applied to lactoperoxidase, the name myeloperoxidase was later adopted. Other leucocytes, particularly eosinophilic granulocytes and monocytes, also contain peroxidases. That of the monocytes closely resembles neutrophil peroxidase and the term myeloperoxidase is currently applicable to the neutrophil and monocyte enzyme (Klebanoff and Clark, 1978: 44).

Mature neutrophils contain very large amounts of MPO, exclusively in the azurophil or primary granules (Bainton et al., 1971; Spitznagel et al., 1974), which are formed at the promyelocyte stage of development in the bone marrow (Bainton et al., 1971). Agner (1941) estimated a content of 1-2% of the dry cell weight, while Schultz and Kaminker (1962) suggested a figure of more than 5%. Venge et al. (1978) found that normal peripheral blood neutrophils contained approximately 40 μg MPO per 10^7 cells. There is a considerable variation in the presence and activity of the enzyme between animal species. For example human, squirrel monkey and dog neutrophils contain large amounts, whereas goat, cat and guinea pig cells have very little, and chicken neutrophils have none (Rausch and Moore, 1975; Klebanoff and Clark, 1978: 45). Enzyme activity

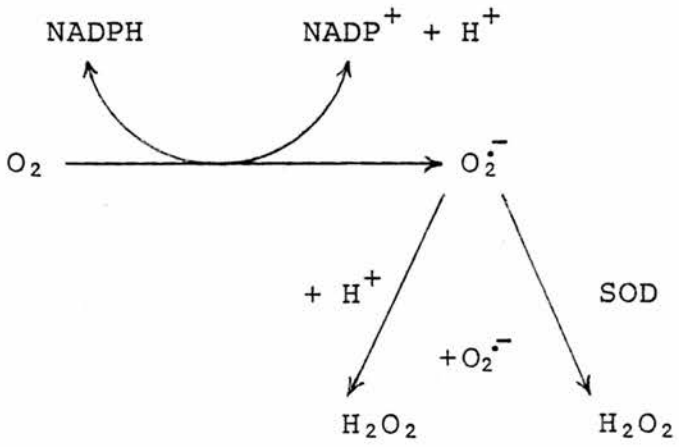
comparisons may not always equate with actual protein (enzyme) quantities. The enzyme activity may be altered appreciably by the extraction procedure, and evidence is conflicting as to the effects of phagocytosis (Klebanoff and Clark, 1978: 45-46). The human enzyme has a molecular weight of 120-144,000 and molecular weights for other species are similar (Klebanoff and Clark, 1978: 46). Myeloperoxidase is a basic protein, the main amino acid being arginine, with two iron atoms constituting up to 0.1% of the molecular weight. Electrophoretic analysis has revealed that the molecule can be divided into separate component parts and speculation as to the existence of distinct isoenzymes has existed for some time (Klebanoff and Clark, 1978: 47). Recently, distinct forms of MPO have been isolated from human neutrophils both by cytochemical (Lippi and Cappelletti, 1982) and chromatographic (Pember et al., 1982) techniques. Although technical artefacts may be responsible for some misinterpretation (Klebanoff and Clark, 1978: 47), Pember, Shapira et al. (1983) have also suggested that the different forms of MPO may exist in separate granule fractions. In human saliva the existence of three distinct peroxidases (not MPO), presumably lactoperoxidases, have been identified (Iwamoto et al., 1972), and recently it has been suggested that two of these may aggregate under the influence of oestrogenic stimulation (Cockle and Harkness, 1983).

Components of the MPO-Dependent Microbicidal System

Satisfactory function of this microbicidal system depends on the presence of MPO, hydrogen peroxide and halide ions. In the original scheme, fusion of the MPO-containing granules with the phagocytic vacuole permits intracellular combination of these agents with bacteria, although by secretory processes or leakage the system may react extracellularly with target organisms (Klebanoff, 1975; Sips and Hamers, 1981). Hydrogen peroxide is produced either by bacterial or leucocyte metabolism.

Bacteria may be classified as either 'producers' or 'non-producers' of hydrogen peroxide. Producers lack the haem enzyme catalase which catalyses the conversion of hydrogen peroxide to water. They therefore tend to utilise flavoproteins for terminal oxidation processes, being devoid of cytochromes (Klebanoff and Clark, 1978: 414). Examples of catalase-negative, and therefore hydrogen peroxide producing microorganisms, are pneumococci, streptococci, lactobacilli and mycoplasma, but there are considerable inter-strain differences in production within a species (Klebanoff and Clark, 1978: 414-415). The hydrogen peroxide formed may contribute to auto-inhibition for that organism, or antagonism towards another species. To the advantage of host

defence, the potential toxicity of the hydrogen peroxide is increased markedly by the addition of MPO and halide ions, which means that the organisms contribute to their own demise (Klebanoff, 1968). Bacterial production of hydrogen peroxide is sufficient for killing by the MPO pathway. This has been shown to occur in classical chronic granulomatous disease (Klebanoff and White, 1969). Hydrogen peroxide is also produced in leucocytes as a consequence of the respiratory burst, as described in chapter 3, and may be summarised as:



These two pathways account for the generation of nearly all the hydrogen peroxide (Root and Metcalf, 1977). Production of superoxide and hydrogen peroxide is centred in the plasma membrane and phagocytic vacuole fractions (Briggs et al., 1975; Dewald et al., 1979; Badwey et al., 1980; Butterick et al., 1983). Also, a recent study has demonstrated considerable hydrogen peroxide production in cytoplasts of human neutrophils, which

contain no nuclear or granular components (Roos, Voetman et al., 1983). Most of the hydrogen peroxide produced is utilised in peroxidatic and catalytic reactions (Romeo et al., 1979). Thus, as was stated in chapter 3, hydrogen peroxide is a significant physiological product of activated neutrophils, and recent evidence suggests that it may be more relevant in vivo than superoxide anion per se (Roos et al., 1982; Curnutte and Tauber, 1983; Roos, Hamers et al., 1983).

The third component of this antibacterial system is halide (Klebanoff, 1967; 1968), the most extensively studied being iodide. In the original experiments iodide was shown to be the most effective co-factor, with a total bactericidal effect observed at a concentration of 5×10^{-6}M (Klebanoff, 1968). The iodination reaction (peroxidation of iodide by MPO with hydrogen peroxide as substrate) was considered to be the most likely basis of the microbicidal mechanism for some years, but reservations have been expressed (Klebanoff and Hamon, 1972; Segal, Garcia et al., 1983). The possible roles of bromide and thiocyanate within neutrophils are as yet unknown (Klebanoff and Clark, 1978: 418-420), but evidence has been accumulating to support the involvement of chloride as the chief co-factor in vivo. Klebanoff (1968) demonstrated the total bactericidal capacity of the MPO-mediated system with

chloride as a co-factor at a concentration of 5×10^{-4}M. The concentration of chloride available within human neutrophils is more than sufficient to permit physiological involvement in the system (Baron and Ahmed, 1969; Morrison and Schonbaum, 1976).

The microbicidal mechanisms are complex and incompletely understood but include halogenation reactions, i.e. the formation of covalent bonds between the halogen and appropriate grouping (e.g. tyrosine) on the organism, or oxidations in which halides are oxidised by the MPO-hydrogen peroxide complex to a variety of microbicidal compounds (Klebanoff and Clark, 1978: 421). Halogenation also seems a probable mechanism for bacterial protein denaturation, although Segal, Garcia et al. (1983) have cautioned that many conclusions are based on extrapolations of theoretical biochemical models. The physiological product of chloride oxidation is thought to be hypochlorous acid (HOCl) (Stelmaszyńska and Zgliczyński, 1974; Zgliczyński and Stelmaszyńska, 1975; Wever et al., 1981), and this is formed over a broad range of pH values (Zgliczyński et al., 1977). The mechanism may be governed by two independently operative chloride binding sites on the MPO molecule (Andrews and Krinsky, 1982a). The efficacy of an hypochlorous acid generating system in neutrophil killing of E. coli has been demonstrated

(Sips and Hamers, 1981) and was found to be effective both extracellularly and intracellularly. Chlorination reactions catalysed by MPO have been demonstrated (Harrison and Schultz, 1976). Recently it was shown that approximately 30% of the oxygen consumed on neutrophil stimulation is utilised in chlorinating reactions (Foote et al., 1983).

Klebanoff and Clark (1978: 425) have summarised a possible reaction for the production of hypochlorous acid:

$$MPO^{-Fe}_{-Fe} + Cl^- + H_2O_2 + H^+ \longrightarrow MPO^{-Fe.HOOH}_{-Fe.Cl} \longrightarrow MPO^{-Fe}_{-Fe} + H_2O + HOCl$$

(Hypochlorous acid is used commercially as a germicidal agent, for example in swimming pools.)

Apart from hypochlorous acid production, chloramines may be formed by oxidation of nitrogenous compounds by the MPO-hydrogen peroxide-chloride system (Zgliczyński et al., 1971; Thomas, 1979). These could be bactericidal in a number of ways, principally by the ultimate release of activated chloride (Klebanoff and Clark, 1978: 426). Soluble aldehydes and singlet oxygen species may also be generated with bactericidal consequences but much of the evidence is theoretical (Klebanoff and Clark, 1978: 426-

428). Recent evidence also suggests that glutathione may be oxidised by the MPO system with microbicidal results (Turkall and Tsan, 1982).

A number of schemes have been proposed for killing of microorganisms by the MPO system but have not been completely elucidated. As for the LPO-dependent system, oxidation of sulfhydryl groups is probable (Thomas and Aune, 1978a). Decarboxylation reactions, lipid peroxidation and the toxicity of hypochlorous acid for bacterial nucleic acids have also been demonstrated (reviewed by Klebanoff and Clark, 1978: 429). Hypochlorous acid contributes to increasing the permeability of cell walls in E. coli (Sips and Hamers, 1981) and this is supported by the work of Thomas (1979). Finally, Albrich et al. (1981) have proposed that the toxicity of hypochlorous acid is directed towards bacterial adenine nucleotides with the subsequent destruction of the electron transport chain.

In summary, the components of the myeloperoxidase-hydrogen peroxide-halide system exist in normal human neutrophils, and sufficient data are available to suggest that it is operative. The relative importance of this microbicidal mechanism with respect to other oxygen-dependent and the oxygen-independent pathways in vivo, however, remains open to argument. Some insight may be gained by a brief consideration of microbicidal activity

in circumstances of deficiency in hydrogen peroxide generation or myeloperoxidase.

Deficiencies in the Myeloperoxidase-Hydrogen Peroxide-Halide System

The literature discussing this system as a host defence mechanism is extensive and the investigation of deficiency states should provide complementary evidence. In experimental circumstances, reduced hydrogen peroxide production is accompanied by decreased microbicidal efficiency, but in a brief review of this subject Klebanoff and Clark (1978: 431-432) emphasised that, as in a genetically-based deficiency state such as chronic granulomatous disease, the killing defect may equally result from inadequacy in other components.

Inhibition of MPO may be produced experimentally by a number of agents, with variable effects on killing. Azide, for example, inhibits MPO and bacterial killing (Klebanoff, 1970). The antithyroid drug propylthiouracil inhibits iodination and killing of staphylococci by human neutrophils (Klebanoff and Hamon, 1972), so illustrating an effect on the halide component of this system.

Naturally occurring (hereditary) human MPO deficiency has received most attention recently. This was first

described 20 years ago by Grignaschi et al. (1963) and a further 13 cases were reported over the following 15 years, suggesting a rare condition. The most quoted of these case reports has been that of Lehrer and Cline (1969), who described a reduced ability of their patient's neutrophils to kill Candida albicans. Many papers and reviews have since quoted this one study as conclusive evidence for the importance of MPO in candida killing. Recent larger studies also support this claim (Parry et al., 1981). It is often overlooked that Lehrer and Cline's patient was also diabetic, as have been others (Cech et al., 1979). This deficiency of MPO, susceptibility to candida infection and endocrine disturbance, has appeared in other conditions such as pregnancy (El Maallem and Fletcher, 1980).

In their review of MPO deficiency to date, Klebanoff and Clark (1978: 711-733) observed that interest in this area was increasing, and so predicted the reporting of more cases. This point was reiterated by Segal (1981) by which time more sophisticated analytical equipment had become available for screening. Parry et al. (1981) found 28 patients deficient in MPO by screening 60,000 individuals, and Cramer et al. (1982) found 45 from approximately 100,000. These figures suggest a total population prevalence of approaching 0.05%, and led Parry's group (1981) to suggest that MPO deficiency may be the commonest functional disorder of human neutrophils.

Several studies have been able to investigate the families involved and uniformly agree on an autosomal recessive pattern of inheritance (Lehrer and Cline, 1969; Cramer et al., 1982; Larrocha et al., 1982).

Many of the studies, whether large or small, report both complete and partial deficiency and a recent investigation has revealed that in partial deficiency the MPO is present in approximately half the normal amount, but is structurally, biochemically and immunologically normal (Nauseef, Root et al., 1983). Perhaps the most striking feature of these studies is the inconsistency between deficiency and susceptibility to infection, and an enormous variation in the severity of infection when present. For example, in the 28 cases identified by Parry et al. (1981) only four were considered to be severely compromised. In some cases killing occurred normally but over a protracted time period (Cramer et al., 1982).

The conclusion to be drawn at this stage is that the MPO-dependent bactericidal and fungicidal pathways are complex and interrelated to other systems of killing. This point was emphasised strongly by Lehrer and Cline (1969) who showed that in four sons of their MPO-deficient proband, the MPO activities were 22 to 38% of the control value and yet all were healthy. Other compensating systems must therefore exist, and it should be remembered

that neutrophils contain very large amounts of this enzyme, which means that a deficiency of 90% may be tolerable before clinical manifestations occur (Harkness, 1981). Finally, careful interpretation of data is necessary since discrepancies may arise between the histochemical identification of an enzyme and activity measurements as determined, for example, spectrophotometrically (Harkness, 1981).

Other Functions of Myeloperoxidase

In addition to the directly bactericidal action of the MPO system, several other functions have been described and these can be classified into two groups. One concerns microbicidal functions, but in which MPO acts by modifying other processes. The other is the role of MPO-mediated reactions in cytotoxicity.

a) The regulatory role of myeloperoxidase

A succession of studies has demonstrated the involvement of MPO in modifying chemotactic factors by neutrophils (Clark and Klebanoff, 1979; Clark and Szot, 1982; Lane and Lamkin, 1983). These studies also showed that the mechanism involves oxidation of sulphur in the methionine residues of chemotactic peptides. A recent

report has further shown that MPO-mediated oxidation of the Fc and C3b receptors on the neutrophil membrane may occur, so affecting particle binding and phagocytosis (Håkansson and Venge, 1983).

Myeloperoxidase may modify the respiratory burst (Segal et al., 1980), and in MPO-deficient neutrophils (Rosen and Klebanoff, 1976; Cech et al., 1979) and monocytes (Locksley et al., 1983) there is increased activity. The prime function of MPO here may thus be to terminate the burst and the production of reactive radicals (Nauseef, Metcalf et al., 1983).

Myeloperoxidase may influence inflammation further in two other ways. Firstly, MPO may inactivate protease inhibitors by a similar oxidative mechanism that operates against bacteria (Matheson et al., 1979; Clark et al., 1981). This could have serious consequences in the pathogenesis of many chronic inflammatory diseases. Secondly, MPO may modify inflammatory and hypersensitivity reactions by the oxidation and iodination of arachidonic acid, thereby affecting subsequent prostaglandin and leukotriene function (Turk et al., 1983).

It may be therefore that MPO has several roles to play in addition to the classical one of direct bacterial killing. Clearly, some of these activities may be protective and others potentially destructive to the host. Recent data have shown that neutrophil MPO may enhance

the release of histamine from mast cells (Stendahl et al., 1983) and the same group had previously implicated MPO in the pathogenesis of dermatitis herpetiformis (Stendahl et al., 1978).

b) Myeloperoxidase and cytotoxicity

The second major field of influence for MPO-mediated oxidations is in cytotoxicity towards mammalian cells (Klebanoff and Clark, 1978: 255-258). A number of cell types have been studied. For example, spermatozoa, in which LPO, MPO or uterine peroxidase with iodide or thiocyanate as co-factor may induce loss of mobility. The hydrogen peroxide may be generated from the neutrophil or by spermatozoal metabolism. Toxicity towards human erythrocytes, platelets and leucocytes has also been demonstrated in a similar fashion, but of particular interest have been the studies of tumour cell sensitivity to MPO-mediated damage. This aspect is included in the above review and reinforced by more recent studies. Clark and Klebanoff (1975) demonstrated tumour cell killing by the MPO system from intact neutrophils, and more recently a similar observation was made with mast cell peroxidase (Henderson et al., 1981). The efficacy of this tumoricidal function is probably related to the extracellular activity of MPO (Clark and Szot, 1981). In fact, the extracellular release of MPO from stimulated

cells was described by Baehner et al. in 1969, and subsequently it was shown that particle ingestion led to a 50-fold increase in the extracellular release of hydrogen peroxide (Root et al., 1975). The efficacy of this system against E. coli has been demonstrated (Sips and Hamers, 1981) and this applies to both prokaryote and eukaryote cell toxicity.

These aspects of neutrophil peroxidase function will undoubtedly receive more attention, but in conjunction with the recent interest in multiple forms of MPO, it has been hypothesised that there may be a link. It is possible that a heterogeneous group of enzymes with similar, overlapping activities are individually able to selectively catalyse oxidations against a broad group of target cells (Kinkade et al., 1983).

Clearly, MPO plays a key role in neutrophil activities and the diverse nature of these (within the context of a single enzyme) may provide part of an explanation as to the apparent over-endowment of each cell. Such an important and widely studied enzyme also lends itself to investigation from another and equally important viewpoint with respect to host defence. That is, the influence of agents including antimicrobial drugs on both the enzyme per se and the enzyme as a marker of neutrophil function.

The Influence of Various Agents on Neutrophil Function and Myeloperoxidase

In purely experimental situations, the most commonly employed inhibitor of MPO is sodium azide (NaN_3), which inactivates the enzyme directly by forming a complex (Klebanoff and Clark, 1978: 432). It has been shown that azide inhibits bacterial killing and this inhibition is absent when MPO-deficient neutrophils are used (Klebanoff, 1970).

More relevant to clinical therapeutics are the possible effects on host defence of agents employed in the treatment of disease. Adverse effects may be so severe as to warrant discontinuation of a drug or so apparently mild as to go unnoticed. Yet in the long run clinically significant problems may be insidiously produced. By contrast, it is possible that a drug may achieve its designated therapeutic effect and simultaneously stimulate some component of the immune system to the benefit of the host. This field has been extensively researched in recent years, and with the continuing development of chemotherapeutic regimes in all areas of clinical practice, will continue to be so.

The effects of many drugs on neutrophils have been documented but this introductory review will be limited to those considered most relevant. The influence of

antimitotic chemotherapy will be mentioned in chapter 5. As examples of differing agents, tobacco smoke has been shown to inhibit neutrophil chemotaxis and metabolism (Bridges et al., 1977; Corberand et al., 1980) and to stimulate the release of lysosomal elastase (Blue and Janoff, 1978). Povidone-iodine compounds, widely used as disinfectants, are toxic to human neutrophils and monocytes in vitro, at concentrations used clinically (van den Broek et al., 1982). The influence of hormones on phagocytic cell function both physiologically and pharmacologically has been studied. Some neutrophil enzymes including MPO may be under oestrogenic control (Cockle and Harkness, 1978; Harkness et al., 1979) and, during pregnancy, neutrophil oxygen consumption may increase (Kvarstein and Gjønnaess, 1981). Steroid drug administration, particularly glucocorticoids, may markedly influence neutrophil function in a manner which is drug and dose dependent (Watson et al., 1979). It is generally agreed that hydrocortisone inhibits particle binding to the C3b receptor (Boxer et al., 1978) and the Fc receptor (Klempner and Gallin, 1978a) of the neutrophil, and that reduced metabolism is secondary to these effects (Forslid and Hed, 1982).

Of particular relevance, however, are the possible harmful or beneficial effects of antimicrobial drugs which are administered as an adjunct to host defences and

are rarely successful in their total absence. A number of reviews have recently appeared, so underlining the importance of this aspect of antimicrobial chemotherapy (Finch, 1980; Mandell, 1982; Raeburn, 1982). Moreover, in 1981 the first international workshop was convened to discuss the effects of antibiotics on host defence mechanisms (Eickenberg et al., 1982). Many studies have employed drugs at concentrations far in excess of those used in practice and others have been performed on animal neutrophils, so that results must be interpreted with caution (Mandell, 1982). The vast majority of studies have been conducted in vitro which provides technical advantages but may not be entirely relevant to in vivo conditions. Among the first reports of an adverse effect on human neutrophils by an antimicrobial drug was that of Lehrer (1971) who demonstrated a reduced fungicidal and bactericidal activity induced by sulphonamides. He presented indirect evidence that the inhibition was directed at MPO, although this finding was not reproduced in vitro. It has been suggested that the sulphonamide inhibition may be irreversible but slow in reacting with its binding site (Harkness, 1972). Many results are difficult to compare and are equivocal. For example, at different concentrations tetracyclines may have no effect on neutrophil functions (Hoeprich and Martin, 1970; Midtvedt et al., 1982) or inhibit phagocytosis (Forsgren et al., 1974) and bacterial killing

(Welch et al., 1981). Both moxalactam and to a greater extent gentamicin inhibit neutrophil chemotaxis but have no effect on phagocytosis or microbicidal function (Burgaleta et al., 1982). Ampicillin in vivo was shown to depress subsequent neutrophil killing of staphylococci in vitro (Raeburn et al., 1976). A newly introduced cephalosporin, AC-1370, enhances neutrophil phagocytosis in vitro (Ohnishi et al., 1983).

Neutrophil chemiluminescence provides an indirect measurement of oxygen radical production and thus killing potential (Allen, 1979) and is being increasingly used to monitor drug effects. Unless standardised, however, it cannot be equated with either phagocytosis or killing but it is an extremely convenient and sensitive assay. The chemiluminescence response is depressed by rifampicin and a combination of trimethoprim and sulphamethoxazole (Siegel and Remington, 1982) but enhanced by the anti-fungal agent amphotericin B (Supapidhayakul et al., 1981).

A further problem in interpretation beyond those already mentioned, is the extent to which the agent penetrates the cell and this has also been an area of controversy (Brown and Percival, 1978; Raeburn, 1982). Once again methodologies, concentrations and cell types have varied so that benzyl penicillin, for example, was shown not to penetrate the phagolysosomes of human neutrophils (Mandell, 1973) or penetrate only slowly

(Prokesh and Lee Hand, 1982). In the latter study, three cephalosporins (cephalexin, cefamandole and cefazolin) also gained only limited access to the interior of the cell. By contrast tetracycline (Park and Dow, 1970), rifampicin and gentamicin (Easmon, 1979) were all readily taken up by neutrophils.

A clinically important consideration stems from a corollary to the above discussion. Bacteria that are phagocytosed and remain viable within neutrophilic or monocytic phagocytes for a considerable time, are protected from the extracellular antibiotic, and especially so if that drug fails to enter the phago-lysosome in sufficient concentration (Solberg and Hellum, 1978; Lam and Mathison, 1983). The interaction between phagocyte, bacterium and antibacterial drug is complex.

Some experimental work has been directed specifically at drug effects on MPO (e.g. Lehrer, 1971). In 1972 Renz et al. reported a 40% reduction in the activity of horseradish peroxidase by ampicillin and 15% by benzyl penicillin, an inhibition that was irreversible. These authors qualified their conclusions by stating that accessibility of the drug to the appropriate site in vivo was unknown, but later demonstrated an in vivo reduction of MPO activity by ampicillin to approximately 70% of the normal value (Harkness and Grant, 1977). A larger study confirmed the earlier observations and further demonstrated

that the reduction in MPO activity by ampicillin was paralleled by a reduction in phagocytosis of staphylococci (Grant et al., 1983). Intracellular killing was not affected. The same study also demonstrated an enhancement of MPO activity and phagocytosis by cefaclor. The antifungal agent, clotrimazole, was investigated similarly and shown to significantly enhance MPO activity in infants suffering from candidiasis (Renz et al.,1974). An interesting point was that the clotrimazole was clinically effective despite attaining serum levels that were not fungistatic in vitro. The absence of an in vitro effect of clotrimazole may indicate a stimulation of synthesis of MPO, and a hormonal control (which has been mentioned previously) was suggested by the in vivo elevation of MPO activity in male subjects given the anti-oestrogenic drug clomiphene (Harkness et al., 1979).

In a recent study benzyl penicillin did not affect the MPO-mediated iodination of staphylococci, and increased killing was the result of non-oxidative pathway stimulation (Root et al., 1981). Other agents that have been shown to affect MPO include dapsone, an anti-inflammatory and antileprotic drug which caused a depression in activity (Stendahl et al., 1978; Anderson et al., 1981). A large number of anti-arthritic drugs inhibit MPO (Matheson, 1982), and several non-steroidal anti-inflammatory drugs inhibit MPO-generated (cell free)

chemiluminescence (Pekoe et al., 1982). Fluoride (Gabler and Leong, 1979) and ascorbate (Anderson, 1981) have also been shown to inhibit the activity of MPO-mediated reactions.

Myeloperoxidase therefore seems to be amenable to study with respect to drug effects as a model of enzyme function in neutrophils. Considering the possible complexities of control of this and inter-related antimicrobial pathways, in vivo studies should prove fruitful.

Assays of Myeloperoxidase

Myeloperoxidase may be assayed for its presence in whole cells by cytochemical methods or for its activity in extracts of whole cells spectrophotometrically.

Until recently, when it was shown that benzidine has carcinogenic properties, the cytochemical method of Kaplow (1965) was widely used in haematological identification of MPO, particularly in the diagnosis of leukaemias. In 1964, Hayhoe et al. described a scoring system for cells on blood films to establish the overall intensity of a cytochemical reaction, so making the assay semi-quantitative. This has been applied to neutrophils stained with benzidine, for example recently in both normal and leukaemic cells (Bendix-Hansen and

Kaspersen Nielsen, 1983a). The leukaemic states will be considered in chapter 5. Graham and Karnovsky (1966) described a similar method using 3,3'-diaminobenzidine (non-carcinogenic) to localise MPO in the azurophilic granules. A recent comparison of the two reagents demonstrated that both were satisfactory with a slightly greater sensitivity being noted for benzidine (Sheibani et al., 1981). Another substrate, p-phenylenediamine and pyrocatechol was also found to be suitable. The Kaplow (1965) method has been used successfully with the diaminobenzidine reagent (Cramer et al., 1982). In 1975, Kaplow introduced a further reagent, chemically unrelated to benzidine, 3-amino-9-ethylcarbazole.

The identification of an enzyme, however, does not provide a measure of its activity and the spectrophotometric assays available provide this information. In principle, addition of the enzyme to a coloured substrate in the presence of hydrogen peroxide allows a continuous assay of the initial reaction, measured as absorbance at an appropriate wavelength. Thus the rate of oxidation is proportional to the enzyme concentration (Babior and Cohen, 1981). Orthodianisidine (Klebanoff, 1965) is a commonly used substrate, and variations on the original method have been described (for example, Cramer et al., 1982). Another benzidine derivative, tetramethylbenzidine, has recently been used for peroxidase quantitation

(Andrews and Krinsky, 1982b) and increasingly used is 2,2'-Azino-di(3-ethylbenzthiazoline-6-sulfonic acid), or ABTS (Shindler et al., 1976). Once again, modifications have been described in an attempt to improve sensitivity (Mäkinen and Tenovuo, 1982).

Lehrer and Cline (1969) used the orthodianisidine method of Klebanoff (1965) in their study of MPO deficiency and their normal values were reproduced in the UK by Renz et al. (1974), and in a later study by the same group (Harkness et al., 1979). Within a laboratory consistency in methodology is essential because there may be small differences between pathways for the various substrates (Childs and Bardsley, 1975).

Neutrophils can now be differentiated from other leucocytes on the basis of their uniquely high peroxidase content (Mansberg et al., 1974), and this system facilitates the identification of MPO deficiency in very large numbers of blood samples (Parry et al., 1981; Cramer et al., 1982). Finally, chemiluminescence assays mentioned previously are being increasingly used in whole cell and cell free systems. Isolated peroxidase, including MPO, can generate light in an experimental system (Puget et al., 1977; Pekoe et al., 1982) and intact neutrophil luminescence depends at least in part on MPO activity (Klebanoff and Clark, 1978: 300-308; De Chatelet et al., 1982; Dahlgren and Stendahl, 1983).

The Present Study

The purpose of this study was primarily twofold. Firstly, to determine the presence and activity of MPO in gingival crevicular neutrophils, and to compare this to known data on peripheral blood neutrophils. Secondly, to investigate the sensitivity of MPO in blood and crevicular cells to antibiotics.

MATERIALS AND METHODS

Determination of Myeloperoxidase in Peripheral Venous Blood and Gingival Crevicular Neutrophils

1. Cytochemical methods

Cytochemical determination of MPO was first conducted on PVB neutrophils from routinely prepared blood films fixed in 10% formalin/90% ethanol, as described by Kaplow (1965). The staining method is described in Appendix 3. The method has been widely used and is simple to perform, so that cytocentrifuge preparations were made of crevicular washings in PBS and treated in a similar fashion.

This pilot experiment was designed to establish the reliability and accuracy of the technique and determine the presence of MPO in crevicular cells in six healthy adults. The preparations were counterstained with Leishman stain or May Grünwald/Giemsa, and the percentage of positively staining neutrophils counted. A semi-quantitative determination of MPO was made using the criteria of Cline (1981), which are based on those of Hayhoe et al. (1964).

For one subject it was possible to repeat the measurements on several occasions including once during

a severe upper respiratory tract infection.

In addition to the above method, several crevicular samples were also stained with the 3-amino-9-ethylcarbazole reagent (Kaplow, 1975). The method is described in Appendix 4. The objective was simply to confirm the presence of MPO as determined by the benzidine method and no counts were performed as above.

2. Spectrophotometric assay

For several years the laboratory in which the MPO activity measurements were made had been using the method of Klebanoff (1965), which is based on the oxidation of orthodianisidine. Having a method so well established for blood neutrophils provided a singular advantage in that a normal range of MPO activity values was available, and those for a new test group would be directly comparable. This method should also be appropriate for the estimation of MPO in crevicular cells.

To determine MPO activity in gingival crevicular and PVB neutrophils simultaneously, blood and crevicular fluid were collected from 30 systemically healthy adult volunteers, 15 male and 15 female. The age range was 17-48 years with a mean of 27.1 years. At this stage the clinical state of the gingival tissues was not specifically recorded.

Preparation of neutrophils: The method was based on that described by Renz et al. (1974). Neutrophils were separated from 6-7 ml venous blood collected into a lithium heparin coated plastic tube to which were added 1.5 ml 6% w/v dextran (molecular weight 200,000, Sigma Chemicals, USA) in PBS, pH 7.2. After thorough mixing, the tube was incubated for 45 min at 4^0C and the leucocyte-rich supernatant fluid removed into conical glass tubes. These were centrifuged for 10 min at 1,500 rpm (approx. 500 *g*) to pellet the cells and the supernatant fluid was discarded. The cell button was resuspended in approximately 10 ml 0.87% ammonium chloride to remove erythrocytes by differential hypotonic lysis. The tube was allowed to stand for 10 min at room temperature and then centrifuged for 10 min at 1,500 rpm (500 *g*) as above. When necessary this step was repeated to ensure complete removal of erythrocytes, as judged visually. The cells (approximately 90% granulocytes) were washed in PBS and resuspended in 0.2 ml acetate buffer 0.01 M, pH 4.0. This was then stored at -20^0C or -40^0C until required for assay.

Gingival crevicular neutrophils were collected in sds, pH 7.2, as previously described. Epithelial cells were removed and gross bacterial contamination reduced by the addition of further saline dextrose and sedimentation at room temperature for 5 min. The 'supernatant' fluid

contained the neutrophil leucocytes which were concentrated into a pellet by centrifugation at 500 *g*, resuspended in 0.1 ml acetate buffer and stored as for the PVB cells.

For convenience, some cell samples were stored at $-40^{0}C$ and others at $-20^{0}C$, which was shown by experimentation not to affect the subsequent assay. In practice, the enzyme assays were performed within a few weeks of cell collection.

Myeloperoxidase assay: The cell preparation was thawed before assay, made up to 0.5 ml with acetate buffer, and subjected to two further freezing and thaw cycles to rupture the cell membranes. Cellular debris was removed by centrifugation at 6,000 rpm and free enzyme activity was assayed from the supernatant fraction. Details of the spectrophotometric assay are given in Appendix 5, and the protein was determined by the Miller (1959) modification of the method of Lowry et al. (1951). All measurements were made with a Pye Unicam SP-8-100 spectrophotometer (Pye Instruments, Cambridge, England). The results were reported as units of activity per µg protein, for blood and crevicular cells from each of the 30 subjects based on a mean of duplicated tests.

Gingival Crevicular Neutrophil Myeloperoxidase Activity and Gingival Inflammation

To investigate the gingival crevicular neutrophil MPO activity further, particularly in the light of finding six samples in which no enzyme activity was detectable in the first experiment, a study group of 30 subjects (15 male, 15 female) was sampled. They had no clinical evidence of gingivitis (G.I. = 0), including the six with no MPO activity. Twenty subjects (10 male, 10 female) with measurable gingivitis (G.I. > 0.5) were also examined. All were systemically healthy. For the non-inflamed group, the mean age was 25.7 years (male 27.7, female 23.6 years) and for the gingivitis group, 26.9 years (male 25.5, female 28.5 years).

A 50 µl sample of the crevicular washings from each subject was diluted with 50 µl PBS in a cytocentrifuge well and a glass slide preparation made for cytochemical staining of MPO (Kaplow, 1965).

Effect of Ampicillin and Cefaclor on Peripheral Blood and Gingival Crevicular Neutrophil Myeloperoxidase Activity

Nine systemically healthy adult volunteers, 5 male and 4 female, with no clinical evidence of gingivitis, but all of whom had demonstrated crevicular neutrophil MPO

activity, were used in this study. None was taking any form of medication. The age range was 23-39 years.

A single 500 mg oral dose of ampicillin or cefaclor was administered between 8 and 9 a.m. on a random double blind basis. Venous blood and crevicular fluid samples were collected immediately prior to drug administration and at specific time points thereafter for 4 hours. Preparation of neutrophils and the spectrophotometric assay of MPO were carried out as described in the previous section.

From the whole blood samples taken, serum was separated and assayed for the level of antibiotic by a standard plate diffusion method at pH 7.4, using Sarcina lutea as test organism. Since cefaclor is inactivated in serum, clotting of the sample was accelerated by the addition of thrombin and stabilised by the method of Broughall et al. (1979). Test samples and antibiotic standards were set up in quadruplicate. The test method was sensitive to 0.1 μg/ml.

Since it was likely that a drug effect on the enzyme activity could be partly masked by normal fluctuations, the crevicular neutrophil MPO levels were monitored 14 times over a period of 22 hours in 1 male subject taking no drugs.

RESULTS

Determination of Myeloperoxidase in Peripheral Venous Blood and Gingival Crevicular Neutrophils

1. Cytochemical methods

The percentage of cells staining positively for MPO and the scores for each of the six healthy subjects are shown in Table 4.1.

TABLE 4.1:

CYTOCHEMICAL DEMONSTRATION OF MPO IN SIX SUBJECTS

		Percentage Positive Cells		Score	
Age	Sex	Blood	Crevice	Blood	Crevice
39	M	78	95	198	299
24	F	89	65	316	126
22	F	91	85	202	160
24	M	86	61	256	82
22	M	95	92	359	264
29	M	75	84	157	131
		80	59	135	90
		83	81	206	164
		-	95	-	299
		99*	88*	369*	179*

*Estimations of MPO during systemic infection.

For one subject several sets of data were available including one determined during an untreated respiratory

infection*. Figure 4.1a shows a peripheral blood neutrophil staining positively for MPO while an adjacent lymphocyte is clearly negative. Figure 4.1b shows a typical appearance of crevicular neutrophils similarly stained from the same subject and Fig. 4.1c illustrates crevicular cells treated with 3-amino-9-ethylcarbazole reagent. All preparations treated with the latter reagent stained positively as illustrated. Thus, overall, a majority of crevicular neutrophis were shown to contain MPO. For the positive cells it was possible to examine the distribution of scores and an example of this is shown in Fig. 4.2 for the subject for whom data were available during health and a systemic infection.

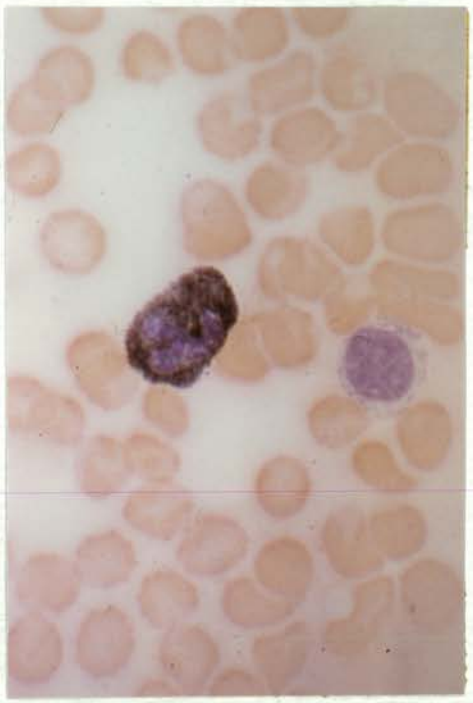
2. Spectrophotometric assay

The normal activity measurements for blood neutrophil MPO expressed as units per μg protein from this laboratory are given in Table 4.2.

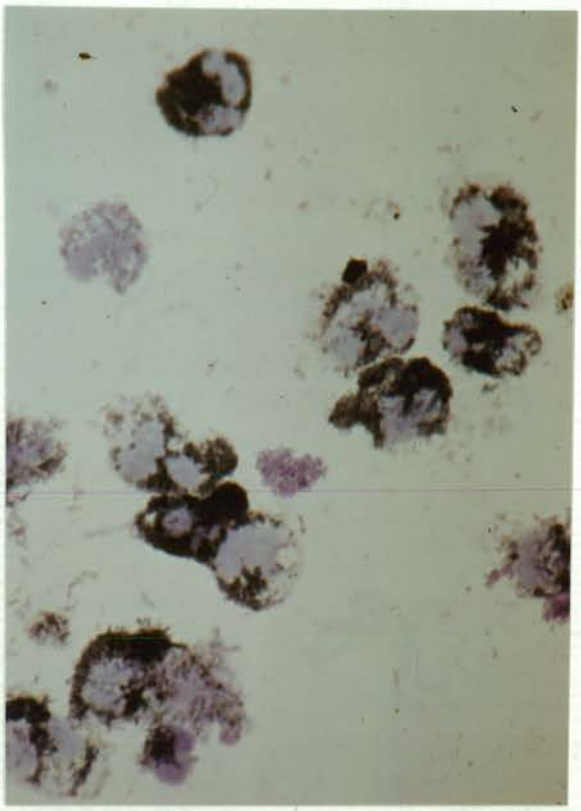
TABLE 4.2:

ESTABLISHED BLOOD NEUTROPHIL MPO ACTIVITY VALUES (units/μg protein)

Source of Neutrophils	Range	Mean ± sem	n
Cord blood	0.7 - 18.5	5.8 ± 1.2	19
Healthy neonates	0 - 42.0	7.0 ± 1.4	80
Healthy children (3-13 years)	0.3 - 8.7	4.7 ± 0.8	32
Healthy adults	2.0 - 7.5	4.5 ± 0.8	44
	(Male	3.9 ± 0.9)	26
(Data supplied by M. Grant)	(Female	5.8 ± 1.8)	18



a



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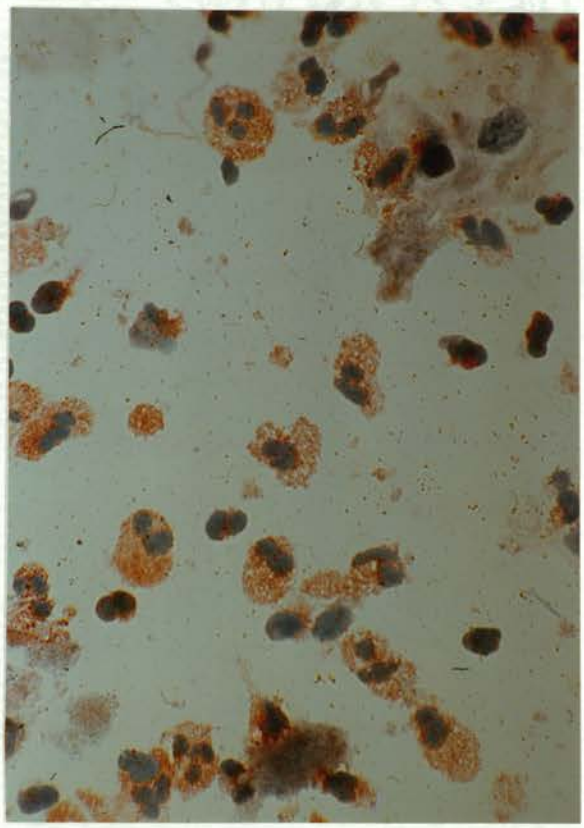

c

FIG. 4.1:

Cytochemical demonstration of neutrophil myeloperoxidase.

a) Peripheral blood film showing a peroxidase-positive neutrophil and a peroxidase-negative lymphocyte. Kaplow's (1965) benzidine method. Giemsa stain. x 1200.

b) MPO positive crevicular neutrophils. Kaplow's (1965) benzidine method. Giemsa stain. x 1200.

c) MPO positive crevicular neutrophils. Kaplow's (1975) 3-amino-9-ethylcarbazole method. Haematoxylin stain. x 750.

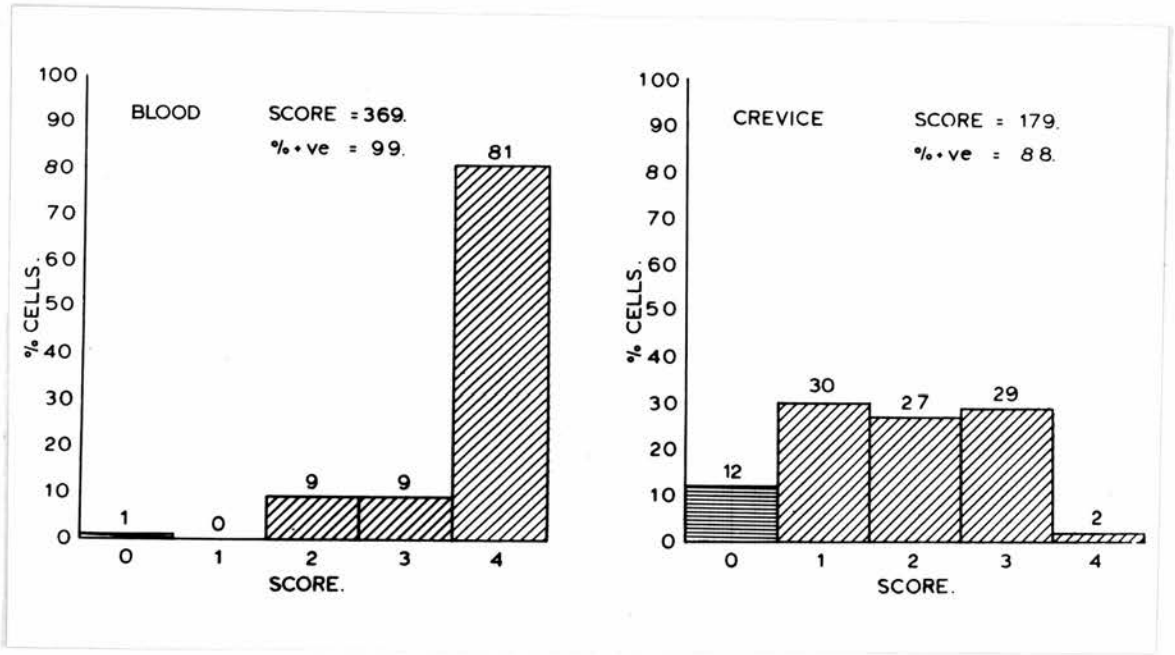

FIG. 4.2: Distribution of scores for cytochemical MPO assay in one adult male subject (age 29 yrs). The test was performed during an upper respiratory tract infection.

The results of MPO investigations from both blood and crevicular cells from the 30 healthy adults are shown in Table 4.3.

TABLE 4.3:

MPO ACTIVITY (units/μg protein) IN PERIPHERAL BLOOD AND GINGIVAL CREVICULAR NEUTROPHILS FOR 30 HEALTHY ADULTS

Female		Male	
Blood	Crevice	Blood	Crevice
2.54	1.77	5.22	1.09
1.93	0.18	2.30	0.39
2.61	0.22	0.21	0.80
11.3	0.30	3.29	2.10
3.99	0	9.0	0.62
3.90	0.92	3.10	0.50
9.20	2.52	1.10	0.52
4.95	1.72	1.40	0
5.19	6.20	3.42	0.62
4.59	0	1.12	0.34
2.79	7.51	4.64	0
6.26	0	2.81	4.65
1.03	0.19	2.80	6.10
1.96	0.20	6.11	2.20
4.90	0	10.5	2.0
Mean 4.47	1.45	3.80	1.46

Note: Values are means of duplicated tests.

In general, the blood values were higher than those for crevicular cells. Five of the blood values fell below and four above the stated normal adult range, although these could not be considered abnormal, particularly without repetition. Six subjects whose crevicular samples had no activity were re-examined and it was found that all had clinically healthy gingivae. For all 30 subjects, the mean blood neutrophil activity was 4.14 and the crevicular cell value was 1.46 units/μg protein. The respective mean values for male subjects were 3.8 (blood) and 1.46 (crevice), and for female subjects 4.47 (blood) and 1.45 (crevice). The total group value accords with the established laboratory figure, and with Student's 't' test, the means for blood MPO between male and female subjects were not significantly different ($t = 1.71$, $p = 0.1$). However, the slightly higher value for female subjects accorded with the existing laboratory data (see Table 4.2). Crevicular neutrophil MPO values for both female and male subjects were virtually identical. To determine any relationship between the crevicular and blood neutrophil values, a linear regression was plotted and the correlation coefficient, r, determined. The r value was 0.034, as determined by the formula given in Appendix 6, and thus no relationship between the values was demonstrated.

Gingival Crevicular Neutrophil Myeloperoxidase Activity and Gingival Inflammation

The crevicular neutrophil MPO activity levels for each of the 50 subjects are shown in Fig. 4.3. The values for all those with inflamed gingivae were scattered within or around the established range for peripheral blood neutrophils. All the samples had measurable activity.

By contrast, the samples from non-inflamed tissues could be divided into two groups. In one, comprising 14 of the 30, the values fell closely in and around the normal range for blood, while in the second group comprising 16 of the 30 subjects, no activity was detectable. This latter group represented just over half the total subjects with no inflammation, and since 10 of the 16 were female, this constituted two-thirds of the female and two-fifths of the male subjects studied.

In all 50 cases, positive staining for MPO was seen on the cytocentrifuge preparations.

The individual MPO measurements of all 50 subjects were plotted against the Gingival Index for each subject and this is shown in Fig. 4.4. The correlation coefficient r for the total group was 0.48, which was statistically significant ($p < 0.01$). When the r value was calculated for the gingivitis group alone ($n = 20$),

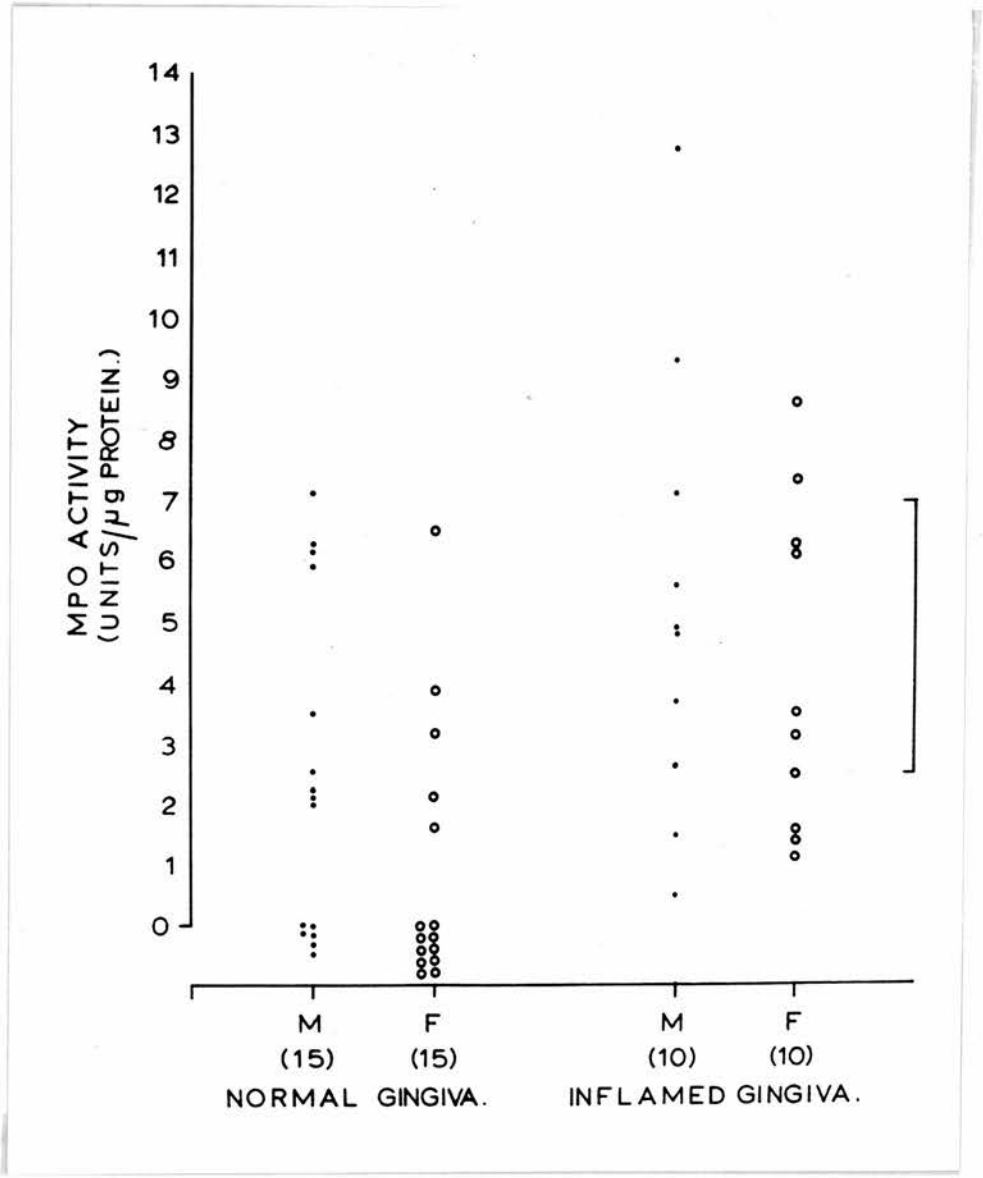

FIG. 4.3:

MPO activity values for 50 subjects with both clinically healthy and inflamed gingival tissues. The normal range for blood neutrophil MPO activity is shown by the vertical bar.

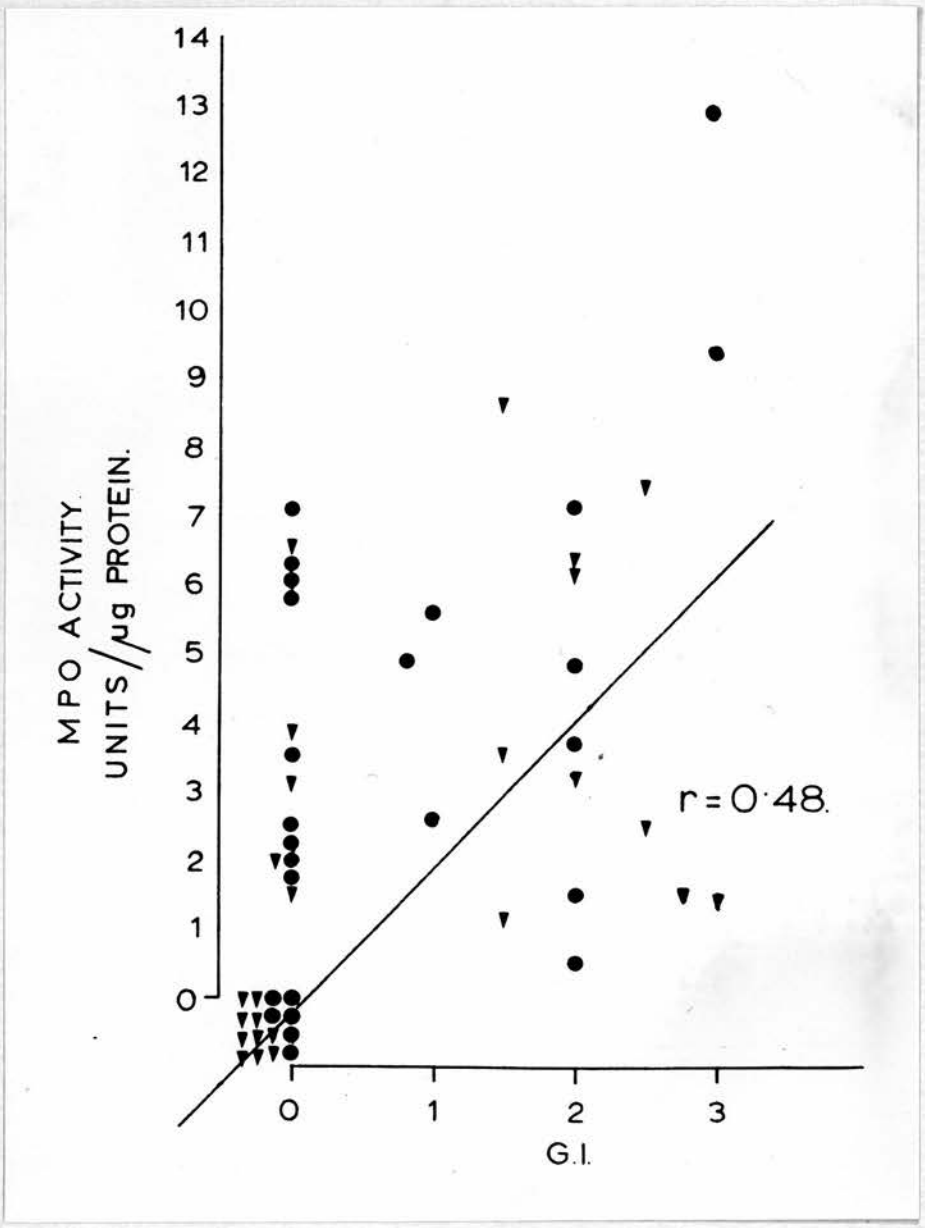

FIG. 4.4:

Relationship between MPO activity in crevicular neutrophils of 50 subjects and gingival inflammation.

● - male

▼ - female

the result was also 0.48 ($p < 0.05$). Thus, increasing inflammation as determined clinically, was accompanied by increasing MPO activity in neutrophils from those tissues.

To investigate any relationship between the results obtained from the group with gingival inflammation and those with perfect health, a non-parametric statistical test was used. The Mann Witney U test is a powerful test for comparing two independent samples, and depends upon initially ranking the values to be compared for both groups. Details of this test are given in Appendix 7. In this case, there were 30 subjects with no gingivitis and 20 subjects with gingivitis.

The value for $U = 126.5$, and $z = 3.436$, therefore $p \leq 0.0003$. Thus the difference between the two groups was highly significant.

Effect of Ampicillin and Cefaclor on Peripheral Blood and Gingival Crevicular Neutrophil Myeloperoxidase Activity

Complete data were available for 7 subjects with cefaclor and 10 (two subjects repeated all experiments and for one data were incomplete) with ampicillin. The MPO values in gingival crevicular cells, monitored in one subject over a 22 hour period are shown graphically

in Fig. 4.5. It can be seen that there was some fluctuation, but this was not marked. Figure 4.6 shows the MPO activity in blood and crevicular neutrophils in one subject as an example, after the administration of ampicillin and cefaclor on separate occasions. As for Fig. 4.5, the values were plotted as log units of enzyme activity. Although in this case there was a marked rise in activity of the crevicular cells with cefaclor, the effect of ampicillin, particularly on the blood neutrophil enzyme, was less easily interpreted. This feature was common to many of the results and for this reason, and because enzyme activities may follow a log-normal distribution, the following analysis was considered appropriate to determine the overall effect of the antibiotics.

The pre-drug level of MPO activity (i.e. at zero time) was taken as the base level. The maximum and minimum levels reached during the course of the experiment were recorded. To determine the overall net change in enzyme activity, the calculation was:-

(log max - log base) - (log base - log min) = x

For each population of cells a mean value was obtained for each drug, and the final value was obtained by taking the anti-logarithm of x. The results were:-

	Blood	Crevice
Ampicillin	-0.12	0.44
Cefaclor	0.18	2.499

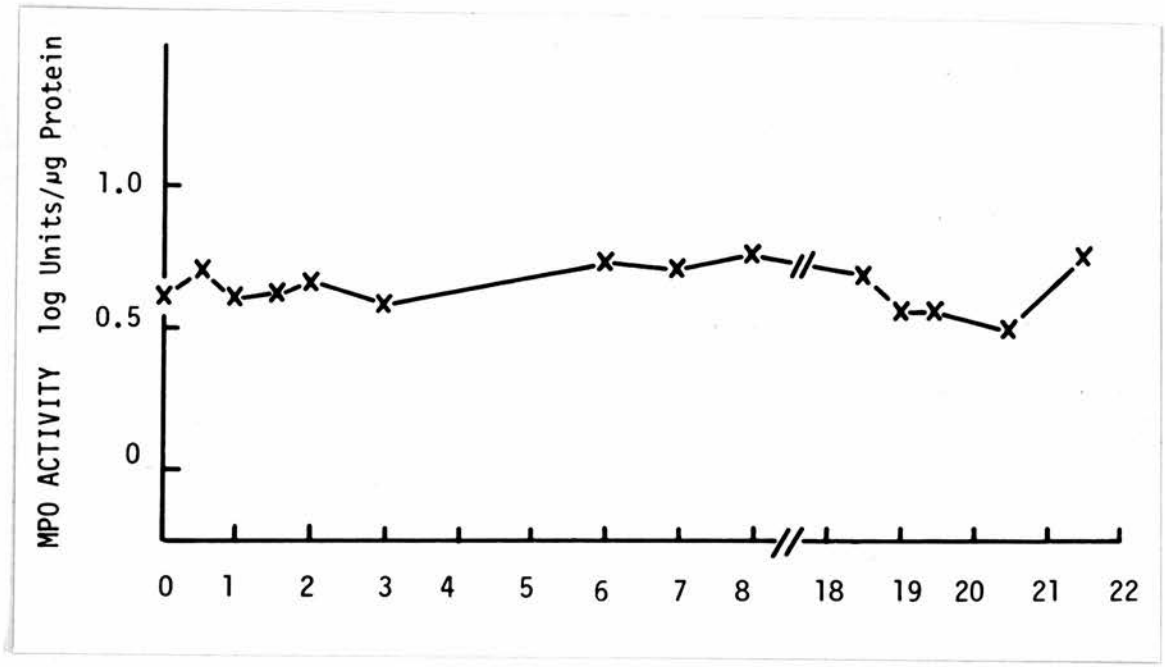

FIG. 4.5:

Gingival crevicular MPO activity measured 14 times over 22 hours in one healthy male subject (age 31 yrs).

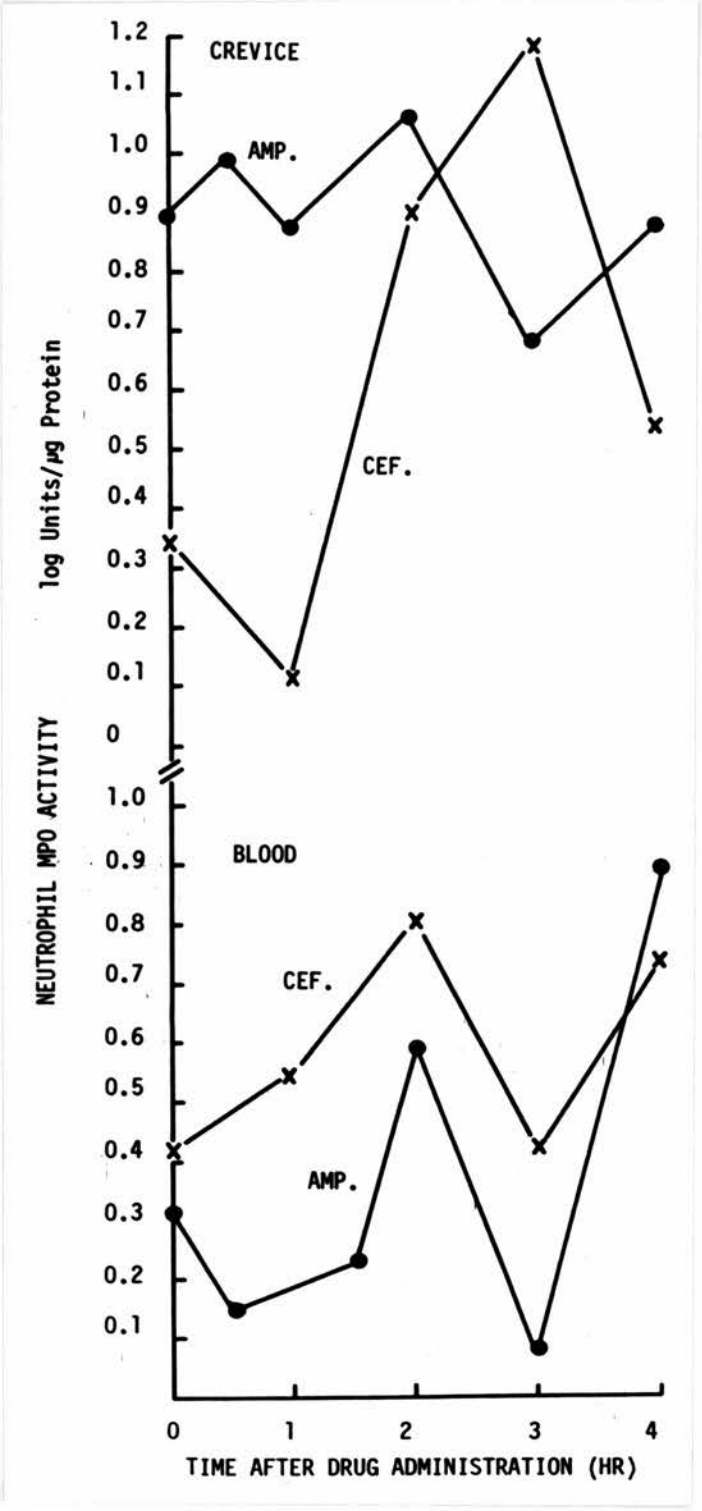

FIG. 4.6:

Effect of 500 mg ampicillin (AMP.) and cefaclor (CEF.) on peripheral blood and crevicular neutrophil MPO activity in one healthy male subject (age 40 yrs).

The negative value for the effect of ampicillin on blood neutrophil MPO demonstrated an overall depression, whilst all other values were positive, implying elevation of activity. This was small, with the single exception of crevicular neutrophil MPO as stimulated by cefaclor. Comparison of the means for elevation with cefaclor for blood and crevicular neutrophils by a paired 't' test revealed a significant difference ($p < 0.01$). No other comparisons produced significant differences.

There was a considerable variation between individuals as to the response seen, and this is illustrated for the total experimental data in Fig. 4.7. The results are plotted as percentage changes in the MPO levels, taking 100% as the base line value. The MPO stimulating effect of cefaclor can be clearly seen and for the total group was at a maximum for the blood neutrophils at 4 hours (subsequent effects were unknown), but for the crevicular cells had clearly reached a peak at 3 hours.

The serum drug assays showed that ampicillin levels reached a maximum in 1-3 hours following ingestion with a mean of 1.5 hours, at a level of 1.45 ± sem 0.27 μg/ml. The cefaclor levels reached a maximum in 1-2 hours, mean 1.5 hours at a level of 9.14 ± sem 1.1 μg/ml. Figure 4.8 shows an example of one experiment in which the peak serum cefaclor level was followed by an increase in the MPO activity, particularly for crevicular cells.

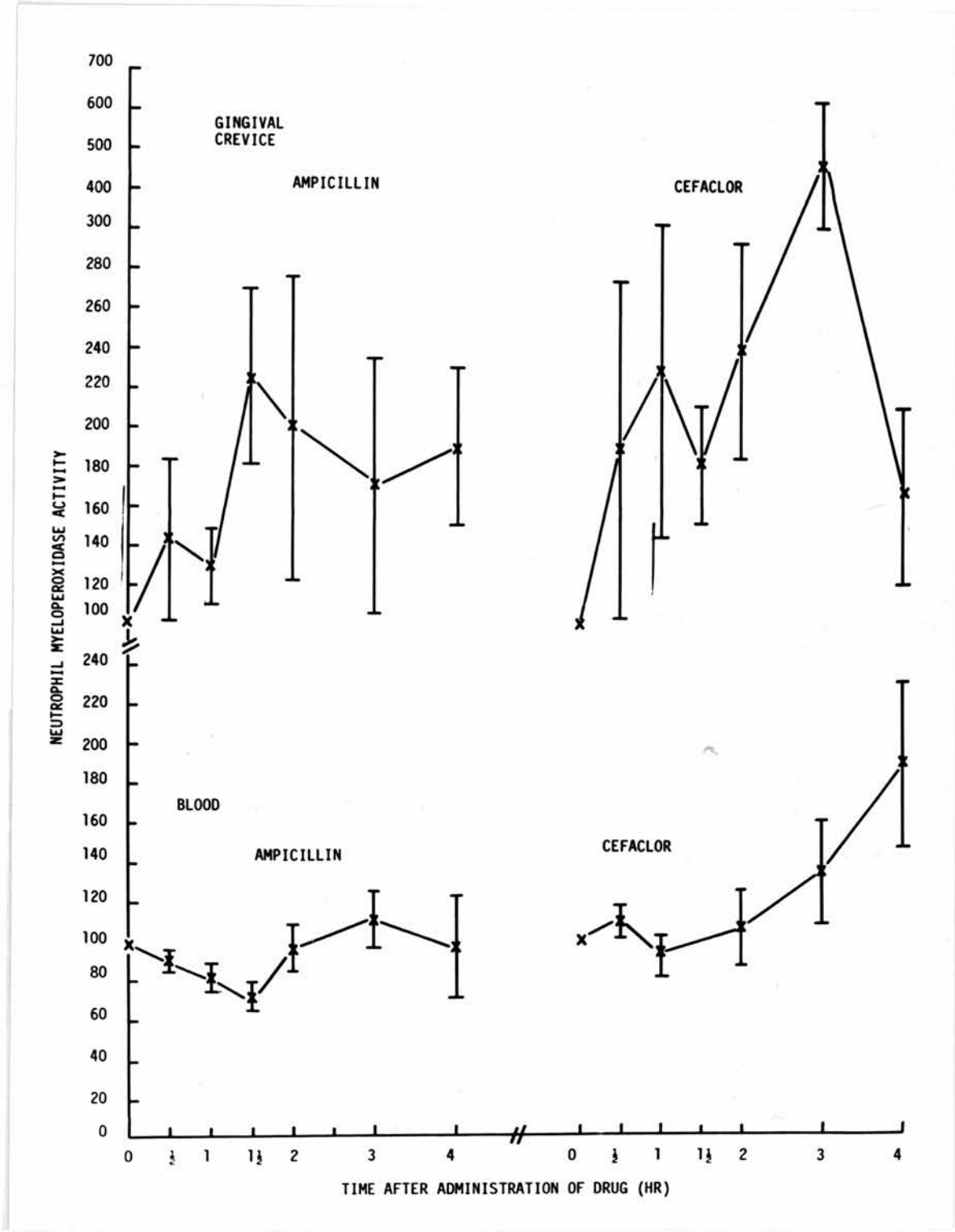

FIG. 4.7: Effect of ampicillin and cefaclor on peripheral blood and crevicular neutrophil MPO activity. The results are expressed as the percentage variation after administration of a single, 500 mg oral dose of drug. Vertical standard error bars are shown. For cefaclor, n = 7; for ampicillin n = 10.

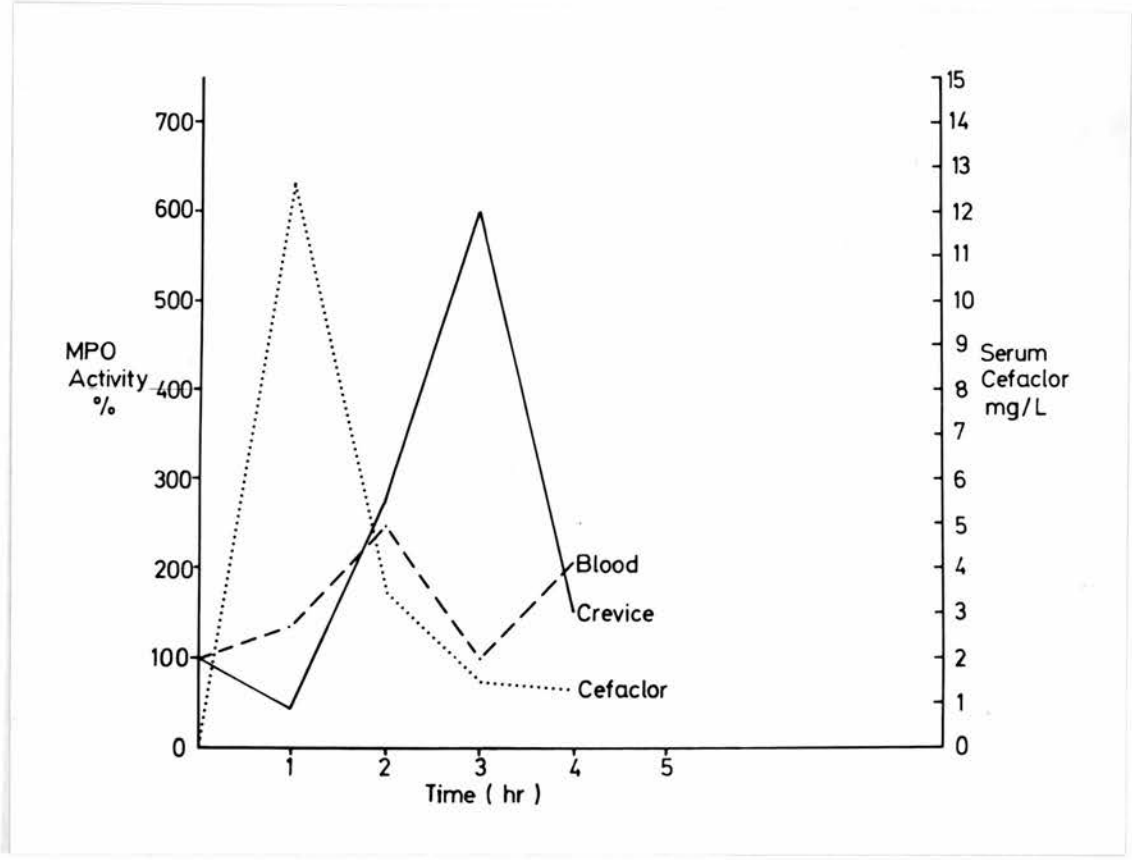

FIG. 4.8: The effect of cefaclor on peripheral blood and crevicular neutrophil MPO activity (shown as percentage of baseline value). The peak serum drug level is followed by an increase in blood neutrophil and then crevicular neutrophil MPO activity.

DISCUSSION

The very extensive literature on all aspects of myeloperoxidase shows the significance granted to this enzyme. In their extensive review of neutrophil function, the largest ever published, Klebanoff and Clark (1978) devoted 60 pages to MPO. Recent studies have suggested that the functions of MPO extend far beyond the classical oxygen-dependent microbicidal activity, and that it is an important regulator of surface receptor activity (Håkansson and Venge, 1983) and the respiratory burst (Segal et al., 1980). The microbicidal efficiency of MPO-mediated oxidations by hydrogen peroxide is also equally effective extracellularly, at least against E. coli (Sips and Hamers, 1981). As if to support these impressive requirements, human neutrophils are heavily endowed with the enzyme (Evans and Rechcigl, 1967; Venge et al., 1978), and yet surprisingly even total deficiency does not appear to lead, in most cases, to increased susceptibility to infection. Indeed, chickens seem to survive well despite being totally MPO deficient (Rausch and Moore, 1975). In man and other species there must be overlapping and compensatory bactericidal systems. Lehrer and Cline (1969) showed that individuals with only 20-30% of the normal MPO complement were healthy, and later showed that neutrophils possessed equally powerful fungicidal cationic proteins (Lehrer et al., 1975). Thus,

deficiency may have to be severe before becoming "potentially" clinically significant (Klebanoff and Clark, 1978: 466-467; Harkness, 1981). The total absence of any granule proteins did not prevent the killing of Staph. aureus in an in vitro system (Roos, Voetman et al., 1983), but in vivo, physical and chemical circumstances differ, and may make more demands on the host.

Myeloperoxidase in Gingival Crevicular Neutrophils

The initial experiments with the cytochemical assay showed that gingival crevicular neutrophils contained large quantities of the enzyme, with a minority of cells giving a negative stain. Although the Kaplow (1965) method of staining is simple to perform, there may have been underscoring, particularly with weak reactions. Harkness (1981) has also pointed out that in some instances MPO activity is detectable spectrophotometrically, even when apparently absent by cytochemical estimation. In the later part of this study, the crevicular neutrophil preparations demonstrated in excess of 80% positive cells for every subject. The small group on whom the cytochemical method was initially tested were not however as homogeneous or constant (one subject) as those of Bendix-Hansen and Kaspersen Nielsen (1983a). The MPO

scores were in the same order of magnitude, but showed more interpersonal variation. As with these authors, staining with the 3-amino-9-ethylcarbazole reagent was quite satisfactory, but required more time and gave preparations on which it was more difficult to distinguish between the less intense degrees of staining.

Since the objective was simply to adopt a technique that might be ultimately applicable to a variety of clinical situations, the benzidine reagent was preferred. From the distribution of scores calculated for one subject (Fig. 4.2), it appeared that in the systemically healthy state there were slightly fewer strongly MPO positive cells in the gingival crevice, although the proportion of positive cells was slightly higher than in blood. With the onset of infection, the blood neutrophil picture altered dramatically, so that 81% of the cells were ascribed a score of 4. By marked contrast, only 2% of the crevicular cells stained so intensely. Since 88% of the latter cells were positive, the most likely explanation is that the majority of cells had partly degranulated in the bacteria-containing inflammatory exudate. An alternative explanation is that by some selective mechanism almost none of the score 4 cells from the blood gained access to the gingival crevice.

While the quantity of an enzyme may be important, especially when below a critical threshold level, the

activity of that enzyme is probably biologically and perhaps clinically, more important. A spectrophotometric assay was employed to measure MPO activity and this method had been well established. The final measurement depends on overall activity; that is, 20% or less of the cells could be very active or 95% weakly active to produce the same result. Nevertheless, this type of biochemical investigation has been widely used, and was employed to study the MPO activity in PVB and gingival crevicular neutrophils from 30 healthy adults. The first encouraging observation was that the blood MPO values were similar to the established mean figures for the laboratory, including the levels for female subjects being higher than those for males. In general, blood values were higher than those for crevicular cells. Two interesting features emerged. The crevicular MPO values for male and female subjects were virtually identical, and six of the samples had no measurable activity. Moreover, no correlation was found for MPO activity between blood and crevice, implying that they are under the influence of independent factors. In the crevice, local factors may influence the enzyme or the gingival cells may belong to a population of neutrophils unrepresentative of those circulating in peripheral blood.

The expanded study gave a clear grouping of crevicular neutrophil results into three categories. The MPO values for all subjects with gingivitis were similar to the normal

range for blood. The values for 14/30 subjects without gingivitis also fell within this normal range. In the third group, comprising 16/30 subjects with no gingivitis, no enzyme activity was detected. This group included those with no activity from the first experiment. Ten out of 15 female and six out of 15 male subjects had no activity. For the inflamed group, the mean activity for the female subjects was 4.17 units and for the males 5.63 units/μg protein. The non-parametric statistical test, the Mann Whitney U test, indicated that the values for the inflamed and non-inflamed groups were quite independent ($p < 0.0003$). This may be explained by some component of the exudate, in the presence of overt inflammation, increasing MPO activity. The lack of correlation between the crevice and blood values seen previously would support that conclusion, as would the subsequent correlation shown between the Gingival Index and MPO activity ($r = 0.48$, $p < 0.01$). It is not possible to deduce from this data whether the MPO activity by enhanced catalysis of oxidation processes might contribute to the inflammatory response or whether the MPO was being influenced by the inflammatory response. Klinkhamer and Mitchell (1979), in the only other experiment of this type, demonstrated a positive correlation ($r = 0.876$, $n = 77$) between salivary leucocyte peroxidase and the rate of cell migration. Cell migration, however, is too variable as a correlate of

clinical inflammation (Hase and Reade, 1979). Several possible explanations exist for finding such a large proportion of negative results in the group with no gingivitis.

1) The Gingival Index is insufficiently sensitive to distinguish two groups of apparently inflamed gingivae. That is, the lack of enzyme activity in one group is a true reflection of non-inflamed tissue, but in the other, activity which is similar to that for the "obviously inflamed" group, indicates subclinical inflammation.

2) The lack of enzyme activity was caused by one or more technical factors. For example, the method was too insensitive, or some natural inhibitor of the enzyme may have been present, although this has not been previously reported. Neither does this explain why the other 14 cases did not behave similarly. Another possibility is that the enzyme from cells in inflamed tissue may be more easily extracted. This seems quite possible since degranulation would be occurring anyway, but does not account for the distinction between the two "healthy" groups, unless a factor as in point 1 is operating.

3) There was no enzyme in the inactive group, perhaps due to total degranulation and extracellular discharge. This is unlikely in very healthy tissues with limited bacterial challenge. In addition, all cytochemical tests were positive, indicating the presence of enzyme at the

stage of cell collection. The cells were not undergoing gross degeneration but the majority were morphologically intact.

4) It is possible that these differences reflect the migration of distinct subsets of neutrophils. Recently it has been demonstrated that neutrophil groups (murine) with different density characteristics contained MPO with differing activity, sensitivity to stimulation and quantity (Pember and Kinkade, 1983). Distinct forms of human MPO may be contained within distinct granule populations in a single cell (Kinkade et al., 1983). The concept of neutrophil subsets or subpopulations has been receiving close scrutiny in recent years, and these have been proposed from several types of investigation. Klempner and Gallin (1978b) and Silvani et al. (1983) identified human neutrophil subsets on the basis of cell surface markers. Two groups of workers have separated cells immunologically on the basis of surface antigen differences with monoclonal antibodies (Ball et al., 1982; Clement et al., 1983). Membrane potential differences (Seligmann et al., 1981) and surface charge density (Brown, 1983) have revealed two sets of human cells (proportionally 83% and 17% in the latter study), and on the basis of chemotactic activity, human neutrophils have also been categorised into two groups (Harvath and Leonard, 1982). Murine peritoneal exudate neutrophils and human blood neutrophils were separated by density gradient

fractionation (Pember, Barnes et al., 1983). Two recent papers are of particular relevance to studies on gingival crevicular cells. On the basis of membrane properties and esterase staining, Edwards et al. (1982) suggested that synovial cells represent a subpopulation of monocytes, and a population of human blood monocytes has been separated on the basis of unusually low peroxidase activity (Akiyama et al., 1983). By electron microscopic examination, the low activity cells were found to contain less enzyme.

The principle of phagocytic cell subsets seems viable and could be applied to the results of this study. Three groups of neutrophil migration patterns, involving two sets of neutrophils, may be hypothesised. In the first group, in non-inflamed tissue, one set of peroxidase-inactive neutrophils is marginating to phagocytose but not kill bacteria or kill by other mechanisms. The second group involves migration of peroxidase-active cells into non-inflamed or subclinically inflamed tissue, and the third includes the same peroxidase-active cells, now migrating into clinically inflamed gingivae.

Following analysis of the results, it was possible to re-check the PVB neutrophil MPO activity in 11 of the 16 subjects who had no activity in the crevicular cells, and all were normal. If MPO deficiency is now considered the most common disorder of neutrophils (Parry et al., 1981),

it would still require the screening of approximately 2,000 people based on published figures (Parry et al., 1981; Cramer et al., 1982) to reveal a true case of MPO deficiency. It is therefore unlikely that one out of the 50 healthy adults studied would have proved to be MPO deficient. These results are subject to several explanations and raise some questions, some of which are open to further investigation.

Effect of Antibiotics on Myeloperoxidase

Just as adverse effects of antibiotics may not be readily distinguishable clinically from the infection itself, so beneficial effects in the normal host may be disguised. The drug's effect on the host is likely to be transient, although any lasting influence particularly adverse is likely to be attributed to the wrong choice of antibiotic in relation to the infecting organism (Raeburn, 1982). It may be that an adverse or beneficial manipulation of defence mechanisms could be relevant to the patient with an evident or subclinically compromised immune response. Thus, if for genetic or other reasons, a defence component is operating at a "borderline" efficiency following infection with a particular organism, treatment with a potentially immuno-modulating drug may push the defences into compromised inefficiency or

conversely into enhanced efficiency.

Ampicillin has been shown to depress human blood neutrophil killing (Raeburn et al., 1976), and MPO activity in vitro (Harkness and Grant, 1977) and in vivo (Grant et al., 1983). In the latter study the effect was associated with reduced phagocytosis, although once internalised the bacteria were killed normally. Staphylococcus aureus was the test organism and it may be that MPO is necessary for killing this bacterium (Roos, Voetman et al., 1983). That is, killing may have been significantly impaired for an organism requiring MPO-mediated oxidations.

Enhanced MPO function has been shown in neonates treated with clotrimazole for oral candidiasis at concentrations of the drug not considered fungicidal in vitro (Renz et al., 1974). Recently, several parameters of neutrophil function (chemotaxis, migration, NBT reduction and microbial killing) were improved in vitro by an experimental cephalosporin (Ohnishi et al., 1983).

The results of this study reflected those above relating to ampicillin effects on blood neutrophil MPO in that the overall effect was depression. For the crevicular cells, the ampicillin effect was variable but overall there was elevation of MPO activity. By contrast, the data from the cefaclor studies were more convincing, and showed an elevation of MPO, both in blood and crevice,

being particularly marked for the crevicular cells. The changes were greater than any normal fluctuation as demonstrated in Fig. 4.5. Several points should be noted. Although the drug dose was identical for each antibiotic, it was evident that the peak serum levels varied, being considerably higher for cefaclor than ampicillin. The reason is unclear but the most likely explanation is a difference in absorption. This is one problem of *in vivo* experimentation, even though it is more relevant to clinical therapeutics. The kinetics of drug effect were observable and are exemplified in Fig. 4.8. A sequential latency between the peak serum drug level, blood neutrophil MPO response and crevicular neutrophil MPO response was seen. A further aspect of this pattern was seen in the early stages following drug administration with cefaclor. As shown in Fig. 4.7 the blood neutrophil MPO activity produced a small peak, followed by a small depression at half an hour. For the crevicular cells, this occurred at one hour. In both cell groups a marked increase was seen and the true maximum may not have been reached at 4 hours for blood, as shown. Quite clearly the crevicular enzyme activity reached the maximum at 3 hours, proportionally greatly in excess of that for blood. This may be supportive evidence for the hypothesis of selective cellular migration as discussed previously. At the least, antibiotic manipulation of cellular components in this manner may provide a useful tool for studying neutrophil migration and function.

The mechanism by which the MPO activity was altered is unknown and may have been direct or indirect. Ampicillin, as other antibiotics, may affect haem enzymes (Harkness et al., 1979). Cefaclor is a chlorinated cephalosporin so that part of the effect may be due to a direct interaction with the MPO-hydrogen peroxide-halide system. The ability of cefaclor to penetrate neutrophils is unknown but in vivo such an interaction could operate powerfully in the extracellular environment.

During infection, neutrophils migrate to the areas of bacterial challenge. If tissue penetration of the antibiotic is satisfactory, then the cells will be exposed to the drug for a protracted period. This in itself could further influence the interaction between host and parasite. Many methods have been employed to study tissue penetration of antibiotics (reviewed by Raeburn, 1978) and several antibiotics including ampicillin (Simon et al., 1978) and cephalexin (Raeburn, 1976) have been shown to enter artificially induced exudates. Moreover, both ampicillin and cephalexin enter gingival crevicular fluid (but not saliva) at concentrations approaching those in serum (Stephen et al., 1980). It is therefore possible that in vivo crevicular neutrophils are exposed to antibiotic effects for longer than the cells sampled from peripheral blood. This could, of course, prove most beneficial in the treatment of oral infections, if the activity of host defence cells can be boosted.

Summary

Gingival crevicular neutrophils have been shown to contain MPO and this is variably active. Several reasons for the inactivity of the enzyme in a proportion of subjects studied who had no clinical evidence of gingivitis have been proposed. Further work is needed to exclude the possibility that this was a technical artefact, but this is unlikely. It is possible that the findings support the hypothesis of selective neutrophil migration, and therefore the published evidence for subpopulations of neutrophils has been reviewed. A positive correlation was shown between the degree of clinically measurable inflammation and MPO activity. Which parameter was the primary influence is open to conjecture.

In view of the widespread use of antimicrobial drugs and the possible effects these may have on host defence mechanisms, two such drugs were studied. Ampicillin increased MPO activity in gingival neutrophils, but depressed activity in blood neutrophils. Such studies may have relevance to clinical therapeutics and to the further understanding of cellular defence mechanisms.

Having determined the functional integrity of gingival crevicular neutrophils in several respects, it was now possible to apply these tests to cells from patients with specific neutrophil abnormalities.

CHAPTER 5

GINGIVAL CREVICULAR NEUTROPHILS IN PATIENTS WITH NEUTROPENIA, LEUKAEMIA AND CHRONIC GRANULOMATOUS DISEASE

THE RELEVANCE OF STUDIES ON NEUTROPHIL DISORDERS

The studies so far have demonstrated a degree of functional integrity in gingival neutrophils which, in combination with evidence of their phagocytic ability (Wilton et al., 1977a; Murray and Patters, 1980; Scully, 1982), must be regarded as valuable constituents of host defence at the mucosal surface. It should be possible to test such a hypothesis by considering the gingival changes in patients who have primary neutrophil disorders or dysfunction secondary to another related disease. Moreover, if neutrophils are important or even critical in the maintenance of health, then measurements of their function either systemically or locally should be relevant for monitoring disease processes.

Many disorders of neutrophils have been identified and described, some in great detail. Congenital neutropenias predispose to chronic and recurrent debilitating infection, while some leukaemic states and their treatment produce life-threatening neutropenia. Neutropenias represent a quantitative host defence defect, but highly specific functional abnormalities have also been reported. MPO deficiency has already been described: a condition until recently thought to be rare, but now considered possibly the commonest disorder of neutrophils (Parry et al., 1981). Nevertheless, MPO deficiency may

go unnoticed clinically. A rare, highly investigated disorder which causes significant clinical infection is chronic granulomatous disease (CGD). Such conditions provide models for the scientific study of normal neutrophil function. With increasing experience and technological sophistication, many other phagocytic cell disorders and variants will be described.

Because these conditions are rare, it was not possible to study large groups of patients. Even leukaemia patients, both in their disease and response to treatment, present individual problems. Rather, the present study explores gingival crevicular cells in neutrophil disorders and the sequential changes therein. The possibilities of monitoring disease and treatment were investigated.

Congenital neutropenia and leukaemias with and without neutropenia were studied and it was also possible to monitor gingival cell changes during granulocyte transfusion. No patients with MPO deficiency were available for investigation but it was possible to study partially some members of two families in whom CGD had occurred.

NEUTROPENIA

Introduction

The term neutropenia defines a state of relative neutrophil depletion in peripheral blood counts. Several hereditary forms have been catalogued by McKusick (1983). In classical cyclical neutropenia, a rare disease which has been recognised clinically for many years (Leale, 1910), neutrophils are produced in, and released from the bone marrow for approximately 21 days, followed by five to seven days of variably depressed production and release (Page and Good, 1957; Guerry et al., 1973). Neutropenias are, however, a heterogeneous group of disorders with differences in aetiology. The cause may be a hypoplastic lesion of the marrow, maturation arrest, i.e. a release failure in which the marrow contains mature cells as well as myelocytes, or loss of mature cells (Kauder and Mauer, 1966; Dancey and Brubaker, 1982). In benign familial (or hereditary) neutropenia, there is no periodicity in production, and patients are often clinically healthy (Cutting and Lang, 1964). Studies have revealed that this condition may also be characterised by a defect in cell release from the marrow (Shoenfeld et al., 1982). The accumulating data that implicate mononuclear cells as prime controllers of

granulopoeisis (discussed in chapter 1) has led to a more detailed classification of neutropenias based on monocyte activity and dysfunction (Chilcote et al., 1983). Other neutropenias may be caused by the presence of anti-neutrophil antibodies, so leading to post-maturation destruction (Boxer and Stossel, 1974; Verheugt et al., 1978; van Leeuwen et al., 1983). In cyclical neutropenia a population of suppressor lymphocytes may be responsible for interfering with granulopoeisis, by inhibiting mononuclear cell production of colony stimulating factor (Verma et al., 1982).

The diagnosis of congenital neutropenia will only be fully elucidated by taking a comprehensive history, clinical examination, sequential peripheral blood estimations, cytological studies in the family and marrow investigations. In some cases other cellular and humoral immune components are altered. For example, there may be a relative or absolute lymphocytosis and monocytosis (Cutting and Lang, 1964). The monocytosis may be quite marked (Page and Good, 1957; Krill and Mauer, 1966), but rarely, monocytopenia accompanies neutropenia (Chilcote et al., 1983). It has been proposed that monocytes may compensate in part for the missing neutrophils (Kay et al., 1976). Other variants include neutropenia with altered immunoglobulin and eosinophil levels (Björkstén and Lundmark, 1976).

The *in vitro* function of neutrophils in neutropenic states has been reported as either normal (Kay *et al.*, 1976) or reduced (Björkstén and Lundmark, 1976). Other conflicting reports have been reviewed by Klebanoff and Clark (1978: 612). This lack of consistency indicates the heterogeneous nature of the neutropenias and the limited number of thorough studies that have been conducted. In each patient there must be a threshold level of neutropenia, possibly indefinable, which may be affected by many factors including the nature of the challenging organism. Quie (1976) has suggested that the development of infection increases with the degree of neutropenia, and that a general threshold level is a count of 1.5×10^9 cells/L in peripheral blood.

The severity of infection in affected individuals varies considerably (Cutting and Lang, 1964; Björkstén and Lundmark, 1976; Kay *et al.*, 1976; Chilcote *et al.*, 1983). In cyclical neutropenia, the patient classically becomes unwell when the neutrophil count drops. Infections may be caused by commensal organisms or opportunists such as *Staph. aureus* or Pseudomonas species, and are typically associated with mucosal surfaces and skin. Some patients thus grow through childhood and adolescence with intermittent hospitalisation and many courses of antibiotics. Oral infections are very common, and ulceration which in some individuals may mark the

periods of neutropenia (Cohen and Morris, 1961; Wade and Stafford, 1963), and in some may be the only clinical manifestation of the disease (Baikie et al., 1967). Gingivitis and periodontitis are often marked, especially in that the deciduous as well as the permanent dentition may be affected (Page and Good, 1957; Cohen and Morris, 1961; Wade and Stafford, 1963; Cutting and Lang, 1964; Davies, 1980; Scully et al., 1982).

Established neutropenias were frequently fatal before the development and widespread use of antibiotics. Treatment is now aimed at controlling infection. It has been suggested that, at least in some cases, the severity and frequency of infections may decrease with age (Kauder and Mauer, 1966). Recently some success has been achieved in stimulating marrow release of cells with systemic corticosteroids (Wright et al., 1978).

In summary, the clinical evidence suggests that, where neutropenia is severe, infection will supervene. The oral cavity is particularly susceptible, perhaps due to the very high number of bacteria normally present. Crevicular neutrophil function was therefore studied in patients with diagnosed neutropenias. These will be discussed as individual case reports.

Case I: Hereditary Cyclical Neutropenia

The first case studied was an 8 year old boy (IS) who had suffered from repeated infections since the age of 8 months. Most of these had been associated with the skin, upper respiratory tract and gums. He was first referred for specialist advice when 3 years old, at which time gingival inflammation and oral ulceration were the primary clinical symptoms. A family study revealed that his father had also been troubled with recurrent infections, particularly oral problems, which had necessitated the loss of all teeth by the age of 21 years. The pedigree is shown in Fig. 5.1. By the age of 7½ years serial studies had shown that the neutropenia in this young boy was cyclical, occurring for approximately one week in three. His paternal grandfather had died from carcinoma of the pancreas but otherwise the family history was not relevant. With the clear history obtained of his father's illness, his mother being healthy, a diagnosis of hereditary cyclical neutropenia (autosomal dominant) was made. A brother (GS), four years his junior, had a history of an above-average frequency of chest infections, and neutropenia has been provisionally diagnosed.

Gingival crevicular and PVB neutrophil studies were performed at the age of 8 and again at 10 years. At the

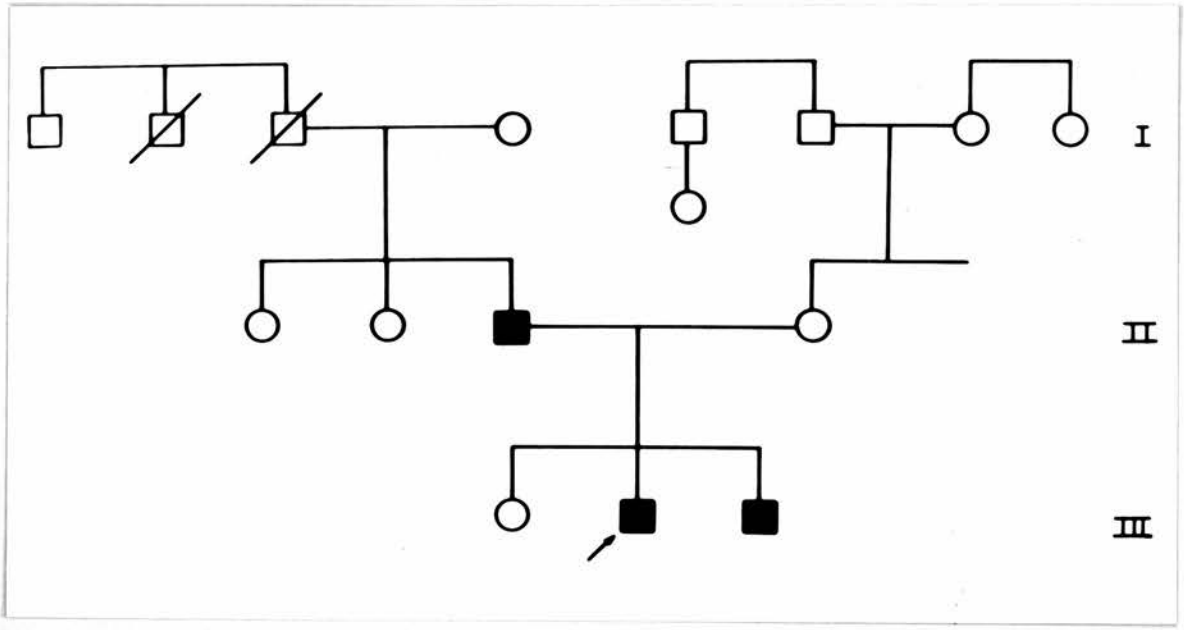

FIG. 5.1: Pedigree of Case I, an 8 yr old boy with hereditary cyclical neutropenia.

second examination studies were also performed on the younger brother (GS), now aged 6 years, and the father (JS) for whom blood values were obtainable.

The results of the first study on IS alone (aged 8 years) are shown in Table 5.1.

TABLE 5.1:

NEUTROPHIL DATA FOR IS, AGED 8 YEARS

	Neutrophil No. x 10^9/L	Differential %	NBT Reduction % +ve Cells	NBT Reduction Stimulated
Blood	1.3*	27*	5.3	65.0
Crevice	0.0021*	59.5*	46.9	-

*Values abnormally low.

The cell counts were low in both cell groups but NBT reduction values were normal. Slightly more extensive data were available for the three subjects tested two years later and these are shown in Table 5.2.

It was interesting to note at this second examination that the gingival condition of IS was extremely severe for a child of 10 years, in that not only was there widespread inflammation but also evidence of periodontitis with alveolar bone loss. By contrast, the oral condition of GS was healthy and at this stage the nature of his deficiency was under review, since he consistently presented with a singular problem of an

TABLE 5.2:

NEUTROPHIL DATA FOR IS, aged 10 years, GS, aged 6 years, and JS (father), aged 36 years

Subject	Source	Neutrophil No. x 10^9/L	Differential %	*In vivo* phagocytosis	NBT Reduction % +ve Cells	NBT Reduction Stimulated	MPO Activity (units/μg protein)
IS	Blood	0.9*	23*	-	0.8	1.1*	1.09*
10 yrs	Crevice	0.037	87	35.9	40.0	-	0.66
GS	Blood	3.9	47	-	1.4	21.5	6.1
6 yrs	Crevice	0.011	100	36.5	35.0	-	0
JS	Blood	0.7*	10*	-	10.3	37.6	2.9

* Values abnormally low.

asthmatic type of complaint, and there was eosinophilia in the peripheral blood. All these male members of the family had consistently shown high levels of serum immunoglobulin, particularly IgG.

Case II: Cyclical Neutropenia

A young boy (DC) aged 2 years had been referred for advice concerning repeated infections and, by the age of 3 years, had been diagnosed as having cyclical neutropenia. In vitro tests for neutrophil function demonstrated normal phagocytosis but bacterial killing was reduced. In this case there was no suggestion of a genetic background, at least of a dominant trait, both parents being healthy and the family history negative. Between the ages of 4 and 5 years, he was clinically well, but by this time a younger brother (GC) had been suffering from repeated infections of the ears and gastro-intestinal tract and from allergic phenomena since infancy. As in the previous family, intensive investigation was difficult, but all four members of the family were seen simultaneously on one occasion and both blood and gingival crevicular neutrophil studies performed. The results are shown in Table 5.3.

Apart from slightly low activity levels of blood neutrophil MPO for DC and his father, all other parameters measured were normal. Both parents had most of their natural teeth with healthy soft tissues, and both boys had extremely healthy mouths, with no gingivitis and below average caries. In the light of the data presented in chapter 4, and the crevicular MPO value of zero for subject GS in the case I report, the absence of detectable activity in the crevicular cells must be construed as

TABLE 5.3:

NEUTROPHIL STUDIES ON FOUR MEMBERS OF ONE FAMILY (CASE II)

Subject	Source	Neutrophil No. x 10^9/L	*In vivo* Phagocytosis %	NBT Reduction % +ve Cells	NBT Reduction Stimulated	MPO Activity (units/µg protein)
DC	Blood	2.8	-	0.7	20.2	1.99
5 yrs	Crevice	0.008	33.2	41.4	-	0
GC	Blood	3.4	-	14.0	41.3	6.61
2 yrs						
IC	Blood	3.0	-	15.0	35.1	1.12
(father) 31 yrs	Crevice	0.02	25.0	39.2	-	0.34
GC	Blood	3.0	-	9.8	28.6	3.42
(mother) 29 yrs	Crevice	0.013	26.8	39.2	-	0.62

being normal. Crevicular studies on the younger brother (GC) in this family were not possible owing to a lack of cooperation.

Case III: Chronic Benign Neutropenia

The third patient was another boy (LO) who had initially presented at the age of 3 years with a severe upper respiratory tract infection which subsequently caused recurrent problems. Haematological monitoring showed that the absolute neutrophil count in peripheral blood was always low, generally fluctuating between 0.2 and 0.8 x 10^9 neutrophils/L. No cyclical pattern was seen but the consistent depression in neutrophils with a corresponding increase in the percentage of eosinophils, monocytes and particularly lymphocytes, provided a diagnosis of chronic benign neutropenia. Both parents were healthy. In vitro neutrophil function tests had demonstrated normal phagocytosis with reduced intracellular bacterial killing. Immunoglobulin (particularly IgG) levels were normal but at the upper limit.

Crevicular and blood neutrophil studies were first performed when LO was 8 years old at which time he was well. There was a moderate degree of gingivitis but oral hygiene was poor and dental plaque levels high to account for this. It was possible to repeat the studies twice, and the results of these are shown as a composite in Table 5.4.

TABLE 5.4:

NEUTROPHIL STUDIES ON PATIENT LO ON THREE OCCASIONS

Age (yrs)	Source	Neutrophil No. x 10^9/L	NBT Reduction % +ve Cells	NBT Reduction Stimulated	Differential %	*In vivo* Phagocytosis %	MPO Activity (units/µg protein)	MPO % (Kaplow, 1965)
8	PVB	2.2	10.4	-	40	-	-	86
	Crevice	0.016	37.6	-	98	36.3	0.6	92
9	PVB	0.62*	1.0	16.3	23	-	1.4*	-
	Crevice	0.017	31.8	-	98	33.1	0*	-
10	PVB	0.78*	7.9	34.5	28*	-	1.77*	90
	Crevice	0.008	41.0	-	100	54	0	94

*Values abnormally low

The circulating neutrophil count was low on two occasions, with concomitantly slightly reduced values for MPO activity. On the third occasion, the crevicular neutrophil count was approximately half that on the previous two occasions, and this was unusual in that an active gingivitis was also present. This may be a reflection of the neutropenia. Crevicular NBT reduction and *in vivo* phagocytosis were highest on this third occasion and yet MPO activity was undetectable. Of interest also in this respect was the normal staining of both blood and crevicular cells for peroxidase.

Conclusions

Although the data for these three case studies are limited, several points merit comment. For all three a diagnosis of the neutropenic condition had been made several years prior to the initial crevicular cell investigations. The first of the two boys (IS) with cyclical neutropenia had a more severe deficiency as evidenced both by the sequential neutrophil counts since diagnosis and the clinical history of infections.

At the first examination of this study both cell groups were reduced in number and a low differential count was seen for neutrophils in the gingival crevice.

This was unprecedented and has not been seen subsequently in this or other neutropenic patients. It is possible that microscopic haemorrhage had permitted efflux of cells from the gingival vessels but there was no evidence of erythrocytes on the cytocentrifuge preparation.

Throughout all investigations in the affected members of these three families, crevicular NBT reduction values were normal, suggesting that whatever the cell number the metabolic activity of the neutrophils was intact. Except for IS on the second occasion, this also applied to the blood neutrophils. It must be remembered that the crevicular neutrophil counts quoted are made from the crevicular washings, and thus do not represent the absolute cell number in native crevicular fluid. The dilution factor is probably of the order of 30 times (Scully and Challacombe, 1979).

The crevicular neutrophil MPO activity values would require further study against a background of age matched control children to understand the relevance to clinical monitoring and management. In the two boys in whom there was no MPO activity, oral health was excellent, but in the third case studied (LO), activity was absent on two occasions, and yet plaque-induced gingivitis was evident. Confirmation of these data is necessary, but they do suggest some inhibition of the enzyme or low levels "ab initio" also seen in part for the peripheral

blood cells. From the three sets of data available for patient LO, it can be seen that the crevicular neutrophil NBT values were paralleled by *in vivo* phagocytosis estimations. However, there is no obvious link between these two measurements and MPO activity, which supports the conclusions drawn from the studies described in chapters 3 and 4.

In summary, these limited studies show that the quantitative and functional studies of crevicular neutrophils are applicable to patients with neutropenia. More extensive work is required to determine if, for example, a strong correlation exists between cell numbers in blood and the exudate, and if differences in the functions of these cells might further indicate any selective migration pattern.

LEUKAEMIA

Introduction

The term leukaemia denotes a neoplasia of leucocyte precursors. The clinical sub-divisions of leukaemia are based on the acute or chronic nature of the disease, and whether the myeloid or lymphocytic series of cells are primarily affected. Diagnosis according to this basic classification determines the subsequent treatment regime. The general features and principles of treatment of the leukaemias will only be discussed briefly.

General Features and Treatment

1) Acute leukaemias

The patient with an acute leukaemia is usually severely ill and symptoms will only have been present for a short time. Malaise, anaemia, fever, bruising and possibly bleeding are the commonest presenting features. Most of these are the result of the bone marrow being dominated by the leukaemic infiltrate at the expense of the normal haemopoietic elements. Gingival bleeding may occur and oral ulceration may be severe, particularly

when there is neutropenia. The peripheral blood white cell count may be normal, low or raised, but the essential feature is the presence of immature forms of either the myeloid or lymphocyte series, particularly blast cells. Distinction between acute myeloid leukaemia (AML) and acute lymphoblastic leukaemic (ALL) is made on the basis of cellular morphology and cytochemical reactions. According to the cell line which predominates, AML can be further characterised as granulocytic, monocytic or myelomonocytic. In AML mature segmented neutrophils may be absent. Thrombocytopenia and erythrocyte deficiency will also be revealed in the blood screen. Bone marrow biopsy confirms the predominance of either myeloblasts or lymphoblasts and a reduction of red cell precursors and megakaryocytes.

Treatment is aimed at inducing a remission of the disease by reducing the output of blast cells, thus hopefully permitting normal stem cell proliferation. Over several years many chemotherapeutic agents have been tested. These include folic acid antagonists, steroids, purine antagonists and alkylating agents which are generally used in combination. The most successful results have been achieved for ALL in children. Acute myeloid leukaemia, particularly in young patients, continues to hold a poor prognosis. Chemotherapy for AML generally requires to be more aggressive than in ALL so that there is an increased risk of serious haemorrhage

and infection. The problem of infection will be discussed subsequently.

In 1970, an interesting adjunct to chemotherapy was introduced in a series of UK clinical trials, based on immunisation of the patients against their leukaemic cells with irradiated autogenous or allogeneic cells, boosted with BCG. Regrettably, long term survival of the patients was not increased and further trials of this type have produced variable results (Alexander and Powles, 1978).

The prognosis of acute leukaemia, particularly the myeloid type, remains poor. Apart from attempting to induce remission, therapy aims at providing as comfortable and as normal an existence for the patient as possible.

2) Chronic leukaemias

Unlike the acute forms of the disease, chronic leukaemias present with milder and more protracted symptoms such as weight loss, tiredness and excessive sweating. As the disease progresses, often over several years, symptoms may become more severe and, for example, chronic myeloid leukaemia (CML) often terminates in an acute phase. Oral symptoms and signs are not usual in the chronic leukaemias. The disease usually presents in patients aged 40 to 60 years, with chronic lymphocytic leukaemia (CLL) being more common than CML.

The peripheral blood picture also differs markedly from that in the acute disease, with the predominant cell being the mature lymphocyte or segmented neutrophil. Immature stages are seen, but are in a minority. Most striking is the highly elevated leucocyte count which frequently exceeds 100 x 10^9 cells/L and splenomegaly is thus very common. A classical cytochemical feature of CML is a low neutrophil alkaline phosphatase score. Bone marrow biopsy reveals a large number of myelocytes (CML) or lymphocytes (CLL), but as in the acute leukaemias, anaemia and thrombocytopenia may be produced by a diminution in the proportion of erythrocyte and platelet-forming components. A classical feature of the vast majority of patients with CML is the loss of a segment of chromosome 22, the so-called Philadelphia chromosome. This segment may be translocated to chromosome 9.

The lifestyle of patients with chronic leukaemia may be virtually normal for long periods. The alkylating agent chlorambucil and/or steroids can be used to treat CLL but, in the absence of symptoms, no treatment may be required for long periods of time. Chemotherapeutic reduction of the leucocyte count in CML allows for recovery of the platelets and haemoglobin concentration, and the alkylating drug, busulphan, has been employed for many years with considerable success. Once the cell count in peripheral blood is within normal limits,

carefully administered intermittent doses can maintain the condition. Because treatment may be continued for several years, side effects of busulphan, for example, may be seen. Many physical and psychological disturbances have been documented but a form of pulmonary fibrosis develops in a small proportion of patients, which may become extremely disabling, much more so than the disease itself. Ultimately, CML may undergo acute transformation or there may be a sudden "blast" crisis. Death is usually from internal haemorrhage, rupture of a splenic infarct or infection.

Infection in Acute Leukaemia and Granulocyte Transfusions

With progressive development of techniques for the efficient collection and replacement of blood components, the problems of haemorrhage in the patient with acute leukaemia can be largely prevented and treated with transfusions of whole blood, packed red cells or platelets. Infection has therefore replaced haemorrhage as the major cause of death (Levine et al., 1972; Chang et al., 1976). The frequency of infections is proportional to the degree of granulocytopenia (Bodey et al., 1966), which is either a result of the overwhelming leukaemic infiltration of bone marrow or the subsequent cytotoxic chemotherapy. Infections are often caused by

endogenous organisms, or by colonisation with Gram negative species such as Pseudomonas aeruginosa or Klebsiella pneumoniae. It is ironical that many of the infections acquired are from contamination by hospital staff, food or equipment (Pizzo, 1983). Non absorbable antimicrobial drugs, laminar flow environments and encouraging "colonisation resistance" by the normal aerobic flora (Young, 1983) have all been used. Unfortunately the antibiotic regimes so widely used may further increase the patient's susceptibility to hospital-acquired pathogenic organisms (Klastersky, 1983; Young, 1983). The combination of trimethoprim and sulfamethoxazole has been used successfully to treat Gram negative bacillary infection (Riben et al., 1983). Approximately 30% of neutropenic patients receiving empirical antibiotic therapy will not respond (Klastersky, 1983) and this underlines the principle that these drugs only function predictably well when the host defences are intact (Glynn, 1983).

In an attempt to supplement the host's defences and provide additional, healthy phagocytic assistance, the other development in recent years has been that of granulocyte transfusion. To achieve successful control of infection, it is necessary to transfuse at least 1 x 10^{10} granulocytes, and since difficulties were initially encountered in collecting sufficient cells, the best donors were patients with chronic myeloid leukaemia (Bodey

et al., 1966; Lister and Yankee, 1978). Subsequently, several methods were developed for the collection of concentrated cells from healthy donors so that relatives of the patient who were HLA compatible might be called upon (reviewed by Lister and Yankee, 1978). Cell collection is not without risk to the donor who must be given anticoagulants, and possibly also corticosteroids (to mobilise as many neutrophils from the bone marrow as possible). Granulocyte transfusion has been partially successful, more so when the neutropenia is severe and the transfusions given in multiple units (Lister and Yankee, 1978).

In a critical review of granulocyte transfusion therapy, Strauss (1978) expressed strong reservations as to its indiscriminate use, on economic as well as clinical grounds. He stressed the paucity of well conducted and controlled studies to date, emphasising that the ultimate success of treatment is bone marrow recovery, which was little influenced by the transfusion of phagocytes. Subsequently other trials were conducted, including one by Strauss' group which concluded from a study of 102 patients, in whom half were transfused prophylactically during chemotherapy, that the risks of transfusion outweighed any benefits gained (Strauss et al., 1981). This conclusion was endorsed by Young (1982) who cited the increasing evidence for cytomegalovirus infections in transfused patients, at least in the United States, and

suggested that the single indication for granulocyte transfusion in acute leukaemia should be as a treatment for infection, once antimicrobial drugs had proved unsuccessful. McCullough (1983) outlined a regime of treatment, including broad spectrum antibiotic administration, intensive hygiene and rigorous identification of the infecting agent, transfusing granulocytes only as a last resort.

It is naturally a prerequisite of granulocyte transfusion that the cells collected are functional, and several studies have produced conflicting results. The general conclusion has been that either continuous or discontinuous flow centrifugation and filtration leukapheresis provide functional cells, with the latter method being slightly superior, and this has been verified by a recent British study (Martin et al., 1983).

Finally, two recent editorial reviews from either side of the Atlantic Ocean have reiterated the regrettable conclusion that granulocyte transfusion, with or without antibiotic therapy, has not been shown to significantly prolong life in patients with acute leukaemia, surely the ultimate goal (Klastersky, 1983; Young, 1983).

Neutrophil Function in Leukaemia

Neutrophil production will be affected either directly or indirectly by the leukaemic state, and function may also be altered by any one of four influences. Already discussed in chapter 4 is the possible influence of the antimicrobial drugs administered. This may well be particularly relevant in acute leukaemia, since the patient is essentially immunocompromised. In this susceptible state, infecting micro-organisms may interfere with host defence mechanisms (O'Grady and Smith, 1981; Mims, 1982). Of obvious relevance is the disease process itself as well as the cytotoxic chemotherapy which will almost certainly affect neutrophil function adversely. Klebanoff and Clark (1978: 601-609) have reviewed in detail the literature on neutrophil function in leukaemia, citing many conflicting and concurring reports. They concluded that clinical infection problems are undoubtedly related to quantitative neutrophil deficiency, but that it is not so easy to generalise when discussing the functional problems, since methods, diagnosis, treatment and stage of the disease all vary, and have not been standardised in the published reports. The relatively inefficient phagocytosis by immature myeloid cells as compared to segmented neutrophils would seem to be an obvious factor predisposing to infection. Metabolic responses are more variable. Neutrophil NBT reduction is

impaired in CML (Whittaker et al., 1974; Pinkerton and Robinson, 1976), AML (Wantzin and Wantzin, 1975) and ALL (Humbert et al., 1976). Patients with AML may have normal, reduced or elevated quantities of MPO (Odeberg et al., 1975; 1976). The deficiency of this enzyme in leukaemia has been specifically reviewed by Klebanoff and Clark (1978: 727-728). In the study of Hayhoe et al. (1964), 10% of patients with AML were MPO deficient, while the proportion was over 40% in a subsequent report by Catovsky et al. (1972). In the latter study the proportion of negative neutrophils in each affected patient was 8 - 70%. Other more recent studies have provided similar conclusions (Cech et al., 1982; Bendix-Hansen and Kaspersen-Nielsen, 1983a). By contrast, in one recently reported case study, AML occurred in a neutrophil population which had highly elevated peroxidase activity (Patterson et al., 1982).

Recent reviews have concluded that most of the anti-cancer agents impair phagocytosis (Mandell, 1982; Pruzanski et al., 1983). For example, reduced granulocyte chemiluminescence, following antimitotic chemotherapy, is thought to indicate drug-induced impairment of microbicidal pathways (Matamoros et al., 1983). In contrast, however, was the finding in the study of Whittaker et al. (1974) that busulphan treatment of CML invariably restored NBT reduction values to normal.

The difficulty in comparing studies has been mentioned, and the altered function seen in vitro may be caused by one or more of the factors operating. Ultimately this is important in two key areas. One is the obvious and critical predisposition to infection, so that increasingly it is becoming possible to carefully balance the types and doses of anti-cancer drugs in relation to disease and influence on host defences. The other is the possible usefulness of monitoring host defence function as a prognostic guide to the leukaemia, as treatment progresses.

Crevicular neutrophil investigations and peripheral blood counts were performed in five patients with diagnosed leukaemia using the techniques previously described.

Case I: Chronic Myeloid Leukaemia

A 57 year old married woman (MRy) attended her general medical practitioner complaining of tiredness, shivering and aching limbs. A routine blood screen revealed an abnormally raised total white blood cell (WBC) count and she was referred for a haematological opinion. On presentation, the WBC count was 187 x 10^9/L, comprising 46% neutrophils, 2% lymphocytes, 2% eosinophils, 7% basophils, 29% myelocytes, 8%

promyelocytes and 6% blast forms. The neutrophil alkaline phosphatase (NAP) score was 30 (low and high controls 65 and 154 respectively). A diagnosis of CML was made. Treatment was instigated with allopurinol, busulphan and 6-thioguanine, with the result that within seven weeks, the peripheral blood picture was near normal: WBC 4.8 x 10 /L, comprising 59% neutrophils, 28% lymphocytes, 2% eosinophils, 1% basophils and 10% myelocytes. The NAP score was 80 (control 48).

The situation was static for two months, when carcinoma of a breast was diagnosed, and surgical treatment was undertaken. The patient became septicaemic in the post-operative phase but this was controlled by antibiotic therapy. Control of the CML was difficult at this time and thereafter 5-8% blast cells were seen in the peripheral blood, despite the chemotherapy maintaining the total WBC and neutrophil counts within normal limits. Mrs Ry died following extensive intracranial bleeding approximately nine months after diagnosis.

Gingival crevicular neutrophil studies were performed sporadically on this patient, the first being an NBT test (result = 38.8% positive cells) performed at the time that the peripheral blood count was initially restored to normal. The gingival tissues were consistently mildly inflamed, and crevicular cells were

readily collected. Crevicular neutrophil MPO activity was assayed on eight occasions and the results are presented graphically in Fig. 5.2. No investigations were possible during the period of surgical care but after this the MPO activity was within normal limits on three occasions. No crevicular assays were performed in the weeks prior to death. At the first examination after Mrs Ry returned to the haematology clinic from the surgical ward, the crevicular neutrophil count was 39,200/ml, cytochemical MPO (Kaplow, 1965) demonstrated 82.1% positive cells, with an MPO activity of 3.1 units/ µg protein, i.e. totally normal values.

Clearly, however, there was a problem of incomplete sampling and thus data collection.

Case II: Chronic Myeloid Leukaemia

The second patient (DN) was a 53 year old married man who complained of weight loss and limb pains. The presenting blood picture revealed a leucocyte count of 97.9 x 10^9/L, i.e. approximately half that of the first patient, and apart from 64% of the cells being segmented neutrophils, the remaining cells were all immature forms. Again, NAP was low and cytology of a bone marrow biopsy showed that 100% of the dividing cells were positive for

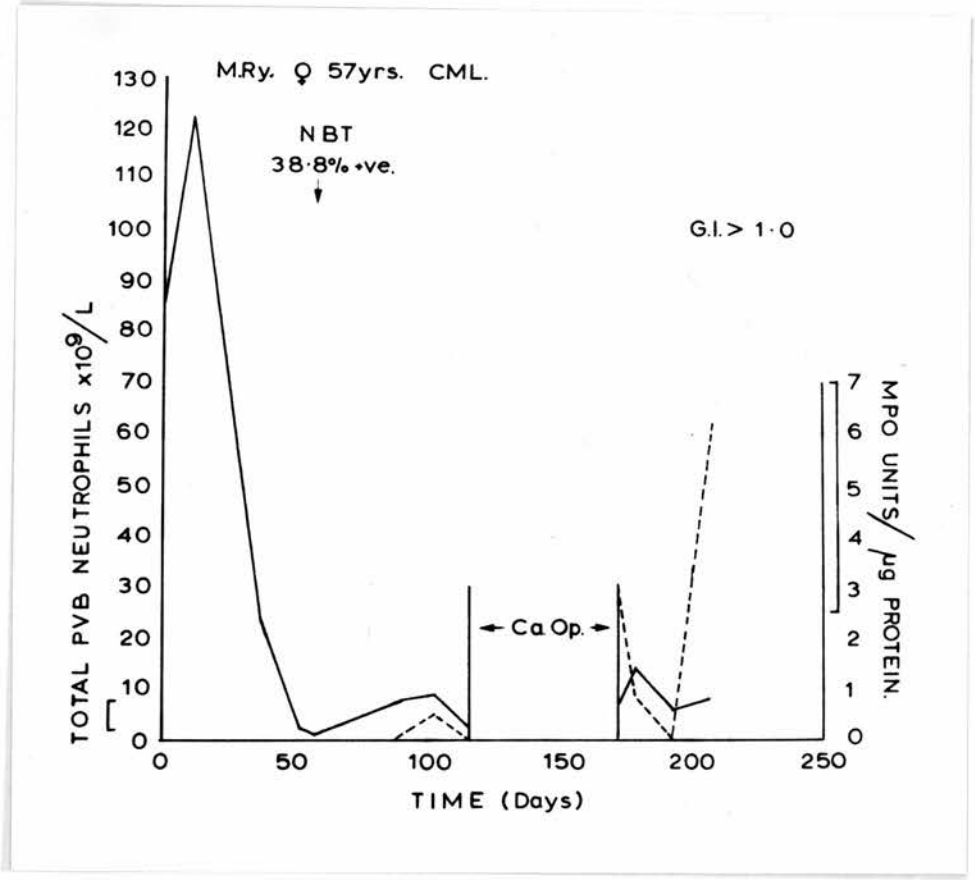

FIG. 5.2: Peripheral venous blood neutrophil count (———) and crevicular neutrophil MPO activity (-----) in a patient with CML (Case I). Ca Op refers to the period during which the patient was undergoing treatment for carcinoma of the breast. Normal values are represented by the vertical bars.

the Philadelphia chromosome. Thus, a positive diagnosis of CML was made. The commencement of treatment with busulphan and 6-thioguanine was delayed pending a report by a respiratory physician, since the patient had a history of chronic obstructive airways disease, and as mentioned previously, busulphan is known to induce serious pulmonary fibrosis in some patients. However, complete drug therapy was started 24 days after diagnosis and the effect on maintaining the PVB neutrophil count over four years until his death is shown in Fig. 5.3. The history throughout was complex but several points should be noted.

In addition to the antimitotic chemotherapy, DN was taking ampicillin or other broad spectrum antibiotics virtually continuously, due to the presence of chronic respiratory infections. These became progressively debilitating but he continued to smoke. Approximately two years after diagnosis, the PVB platelet count started to rise dramatically and control was attempted, unfortunately with little success, by steroid drugs and plateletpheresis. The serum alkaline phosphatase rose steadily and reached 3,210 units/L on the day of his death (normal level 30-140 units/L). The CML thus terminated in acute blast crisis. Post mortem in addition revealed a perforated duodenal ulcer and peritonitis. Splenomegaly and right ventricular

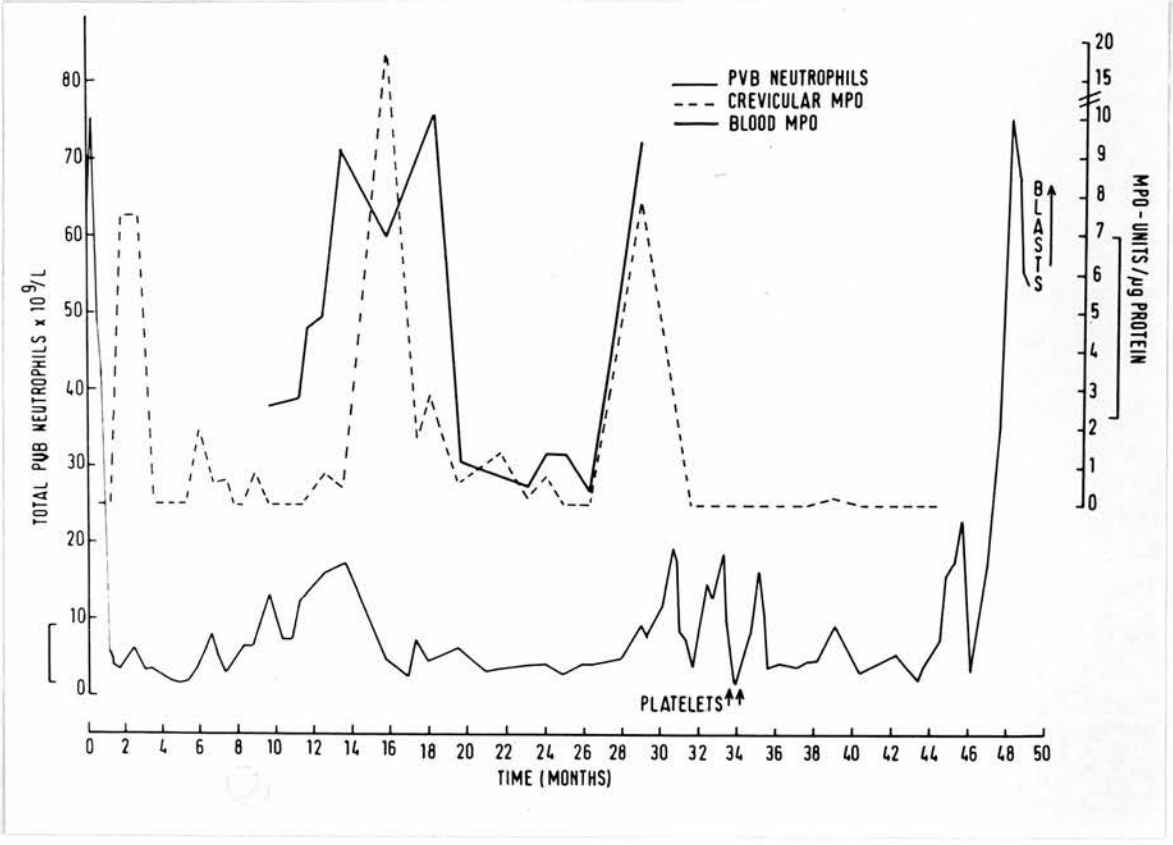

FIG. 5.3: Circulating neutrophil count and MPO activity in peripheral blood and crevicular neutrophils in a patient with CML (Case II), over 4 years. Vertical bars represent normal values.

hypertrophy (associated with the respiratory disorder) were marked.

It was possible to monitor the crevicular cells throughout the course of this patient's disease, from two weeks before treatment commenced until several weeks before his death. The results of 36 crevicular neutrophil MPO activity measurements are shown with the PVB neutrophil counts in Fig. 5.3. While enzyme activity was detectable for most of the four year period, the values were generally below the normal range for blood neutrophils. There were three occasions on which the values were high. The first increase was associated with a reduction of PVB cell counts to normal by treatment. The second peak did not coincide with other obvious changes. The third coincided with the beginning of erratic marrow production of neutrophils, and thrombocytosis. During 19 months, 13 measurements of PVB neutrophil MPO activity were made. These are also depicted in Fig. 5.3 and show an extremely variable pattern.

A number of other crevicular neutrophil functions were measured. The NBT reduction value after the commencement of treatment was 42.7% positive cells. The differential cell count performed on three occasions was also normal, with 98%, 96% and 99% neutrophils. The total neutrophil counts varied between 8,330 and 25,100

cells/ml of washings but were not made prior to treatment or in the terminal stages when the peripheral blood values were so high. At the time of starting treatment, the crevicular NAP was determined and the results for the percentage positive cells and distribution of scores are shown in Fig. 5.4. The values for a healthy male subject measured on the same day are shown adjacent to those for DN. The peripheral blood NAP score of the patient was 22 (controls 100 and 140) as determined by the routine haematological screen. Thus, the crevicular neutrophils demonstrated a marked reduction in NAP, which is in accordance with the typical findings in CML. The results of cytochemical determinations of crevicular MPO on three occasions are shown in Table 5.5. Before treatment started (day 10), the value was low and then increased to normal as the peripheral blood picture improved.

TABLE 5.5:
CYTOCHEMICAL (QUALITATIVE) MYELOPEROXIDASE IN GINGIVAL CREVICULAR NEUTROPHILS FROM A PATIENT WITH CHRONIC MYELOID LEUKAEMIA (Case II)

Day	% +ve Cells	Score
10	48	67
38	56	84
73	88	185

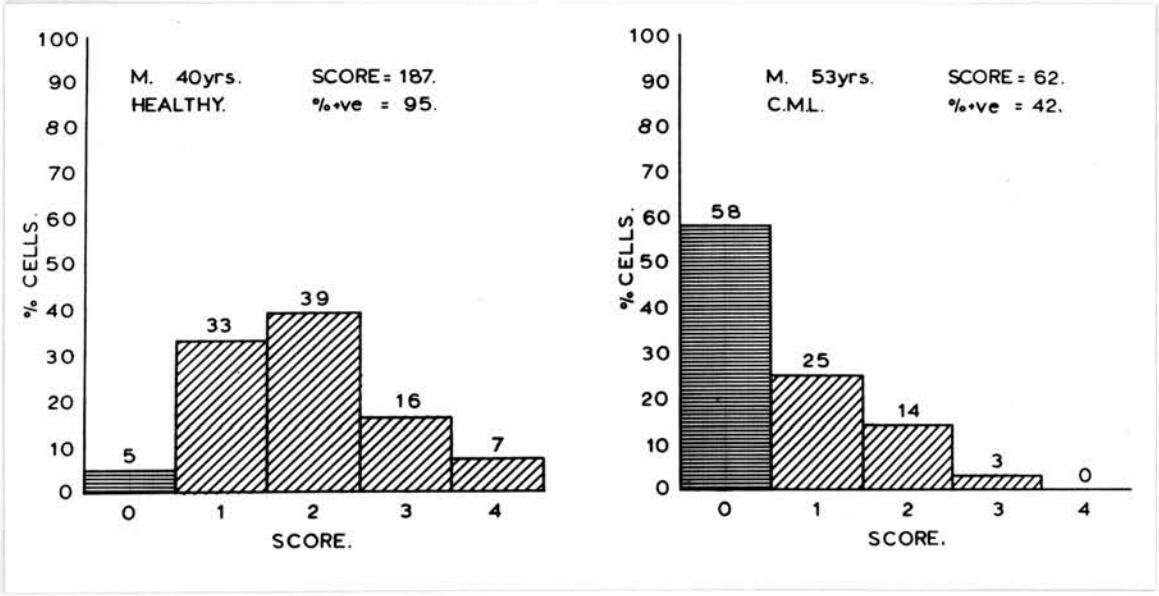

FIG. 5.4: Distribution of crevicular NAP scores in a patient with CML (Case II) and a healthy adult control subject.

Case III: Acute Myeloid Leukaemia

A 25 year old married woman (FO) was referred with symptoms of weight loss, appetite loss, tiredness and a diagnosis of anaemia which had not responded to treatment. The presenting blood picture showed a total WBC count of 16.8 x 10^9/L, with a differential count of 10% neutrophils, 14% lymphocytes, 6% monocytes, 9% metamyelocytes, 16% myelocytes, 21% promyelocytes and 24% blast cells. The serum lysozyme level was also high. Following cranio-spinal irradiation, courses of chemotherapy were used to control marrow activity. In fact, within two months the blood picture was essentially normal, with a total WBC count of approximately 3 x 10^9/L and no blast cells present. The treatment used was the so-called TRAMPCOL regime consisting of thioguanine, daunorubicin, cytosine arabinoside, methotrexate, prednisolone, cyclophosphamide, vincristine and L-asparaginase. Unfortunately she suffered an acute phase relapse one year after diagnosis, and died several weeks later after a massive intra-abdominal haemorrhage.

It was during this latter phase, while undergoing a course of chemotherapy, that she was first seen for crevicular neutrophil studies. At that time, the gingival tissues were cyanotic but otherwise little inflamed. The crevicular MPO activity was determined on five occasions, and the values are shown in Fig. 5.5

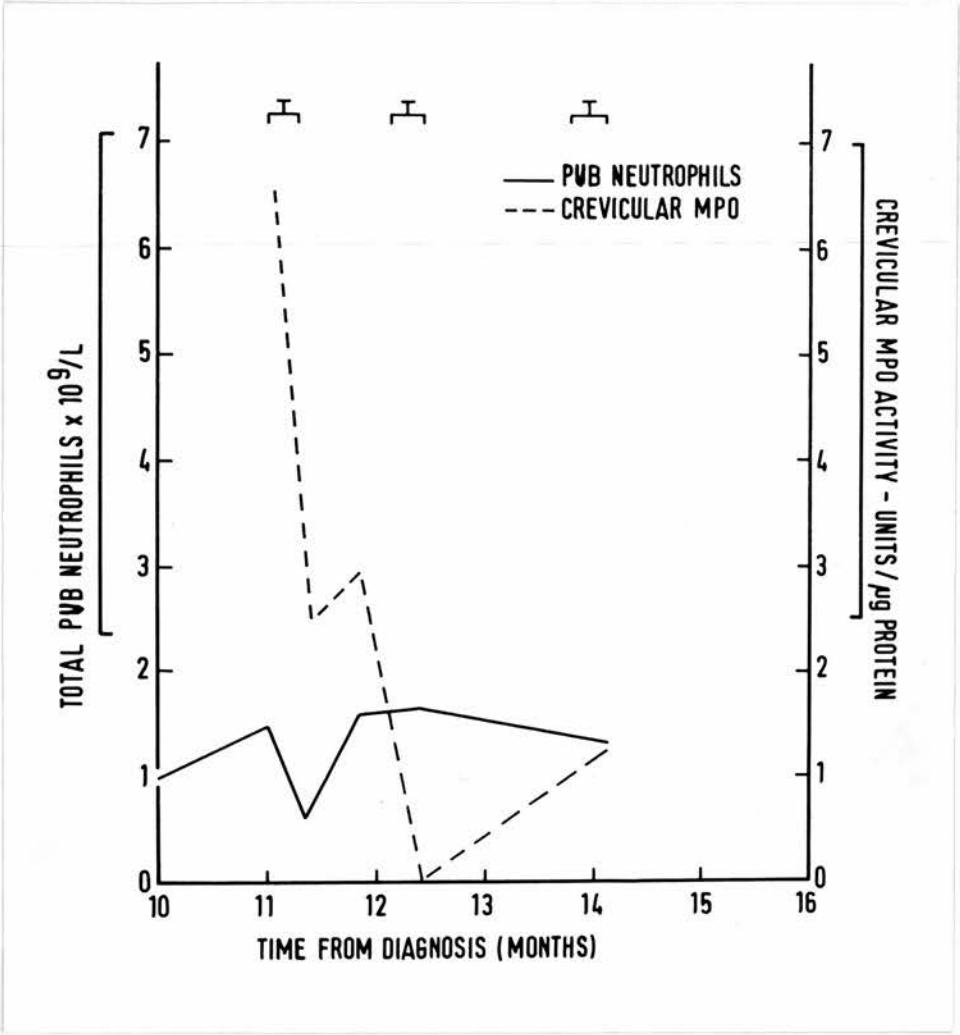

FIG. 5.5: Peripheral venous blood neutrophil count and crevicular neutrophil MPO activity in a 26 year old female patient with AML (Case III). The bars marked T represent periods of chemotherapy.

against the PVB absolute neutrophil count. Two points are clear from these limited data. Firstly, Mrs O was neutropenic throughout the observation period but neutrophils were present in the gingival crevice. Secondly, there was an indication that the chemotherapy (marked with bars on the graph as T) was influencing the crevicular MPO activity. On three occasions MPO was assessed cytochemically, and the results are shown together with the appropriate MPO activity in Table 5.6.

TABLE 5.6:
CREVICULAR NEUTROPHIL MYELOPEROXIDASE IN ACUTE MYELOID LEUKAEMIA (Case III)

Cytochemical		Activity
% +ve Cells	Score	(units/µg protein)
100	261	6.55
97	362	0
90	282	1.2*

*Crevicular neutrophil NBT test - 7.5% positive cells.

Despite low activity level on one occasion and no activity previously, the neutrophils stained very strongly for the enzyme indicating that neither the disease nor the chemotherapy were preventing its synthesis. At the time of the last assay the crevicular neutrophils

demonstrated diminished NBT reduction.

At no time were blast cells seen in the crevicular washings, although present in peripheral blood (for example, 38% leucocytes were blasts when the crevicular neutrophil MPO activity was 6.55 units/μg protein). This crevicular cell observation was confirmed by a haematologist.

Case IV: Acute Monocytic Leukaemia
(monitoring Granulocyte Transfusion)

Acute monocytic leukaemia (AMøL) was diagnosed in a 15 year old girl (MR) who had been complaining of a sore throat and rash, with fever. There was generalised lymphadenopathy, and her blood screen showed severely reduced haemoglobin with neutropenia. A course of radiotherapy was followed by cytotoxic chemotherapy in a similar manner to the previous patient, with intermittent combined antimicrobial therapy as necessary. For three months she was able to live at home for most of the time, but then suffered a relapse and was admitted to hospital with a WBC count of 12.9 x 10^9/L, comprising a differential of 41% lymphocytes and 56% blast cells. No segmented neutrophils were seen. The day of admission was taken as time 0 for the purpose of monitoring subsequent progress. Five days of chemotherapy (in a laminar flow

unit) produced a WBC count of 0.8 x 10^9/L mostly lymphocytes with 2% neutrophils and no blast cells. After ten days, during which no neutrophils appeared in the circulation, a further course of chemotherapy was given. One week later the WBC count was 0.3 x 10^9/L and consisted entirely of lymphocytes. She became febrile and extremely ill. Two days of aggressive combined antimicrobial therapy did not reduce the fever (T = 39.8^{0}C) and a viridans streptococcus was cultured from the blood. Two successive granulocyte transfusions, using the most suitable HLA matched donor, her 17 year old brother, were then given. Granulocytes were collected using a continuous flow cell separator, 2 units of cells (1.9 x 10^{10} cells/unit) being transfused with a 17 hour interval between. This treatment successfully overcame the infection and the patient's own marrow subsequently showed signs of recovery (see Fig. 5.6). Between days 45 and 60, however, the neutrophil count started to fall and the reappearance of blast cells was confirmed. A "maintenance" regime of chemotherapy was introduced, but the patient became acutely ill and, despite the reintroduction of more aggressive therapy, died from an intracranial haemorrhage. At this time the serum lysozyme level was 280 µg/ml (normal range 3-12 µg/ml), so indicating a massive breakdown of cells.

The principal investigations of crevicular neutrophils in this young girl were quantitative. The cell count was

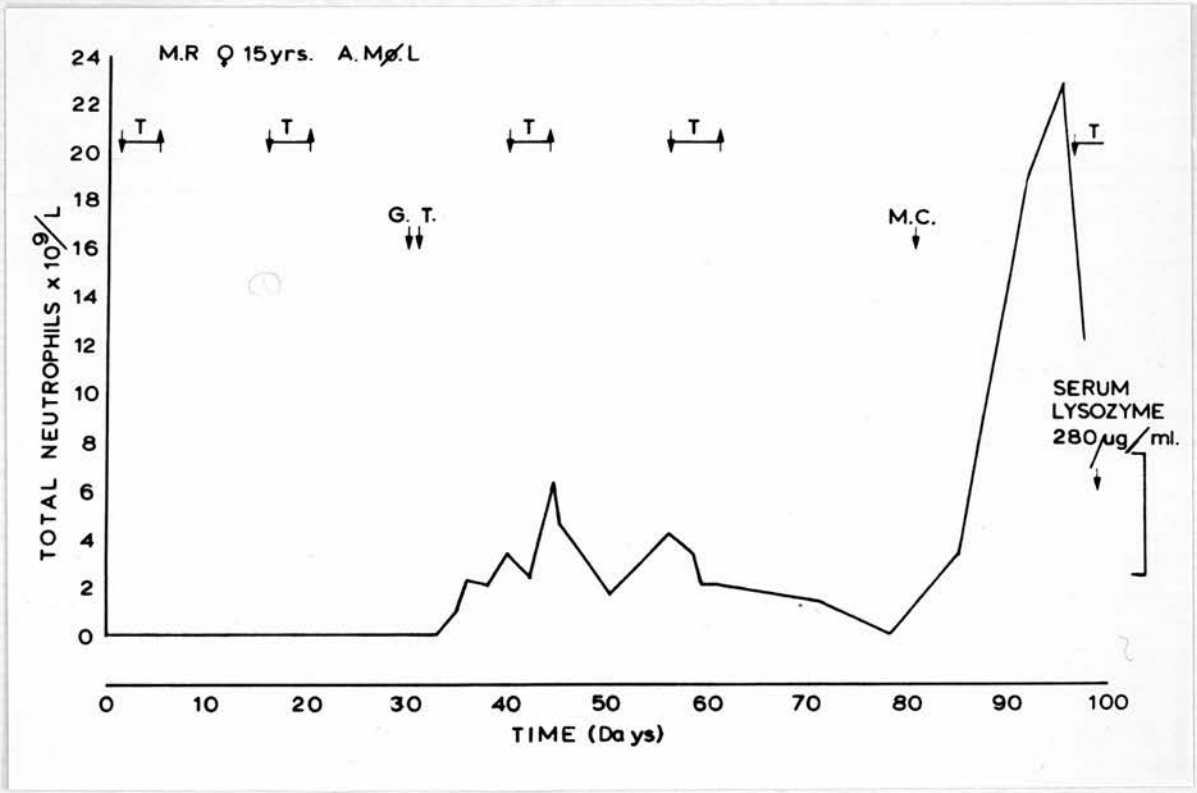

FIG. 5.6: Absolute PVB neutrophil count in a 15 year old female patient with AMøL. Bars marked T indicate periods of chemotherapy. G.T. = transfusion of two units of granulocytes. M.C. = period of 'low dosage' chemotherapy. The vertical bar indicates the normal circulating neutrophil count.

monitored just before, during and after the granulocyte transfusions, and the results are shown in Fig. 5.7. The time scale has been adjusted so as to demonstrate the change in neutrophil counts, shown logarithmically. The PVB neutrophil count is also shown, and none of the transfused cells was seen in the circulation. The most likely explanation is that the cells, on transfusion, were immediately sequestered into the areas of bacterial challenge, primarily the lungs, but to some extent into the crevicular domain. Other cell preparations were stained for MPO with the Kaplow benzidine reagent and an estimate of _in vivo_ phagocytosis was also made from the cytocentrifuge slides. The results of these two measurements are given in Fig. 5.8, and show a close parallel with the cell counts.

A sample of cells was taken from the empty bag following the second transfusion. They were assayed for MPO cytochemically and shown to be 83.2% positive. This test was performed approximately eight hours after the cells had been harvested from the donor and irradiated. Not surprisingly, the blood film made from the donor cells showed a marked 'left shift' in the neutrophils.

Prior to the transfusions, sufficient crevicular cells were obtained to assay MPO activity on two occasions. Subsequently 12 further assays were performed

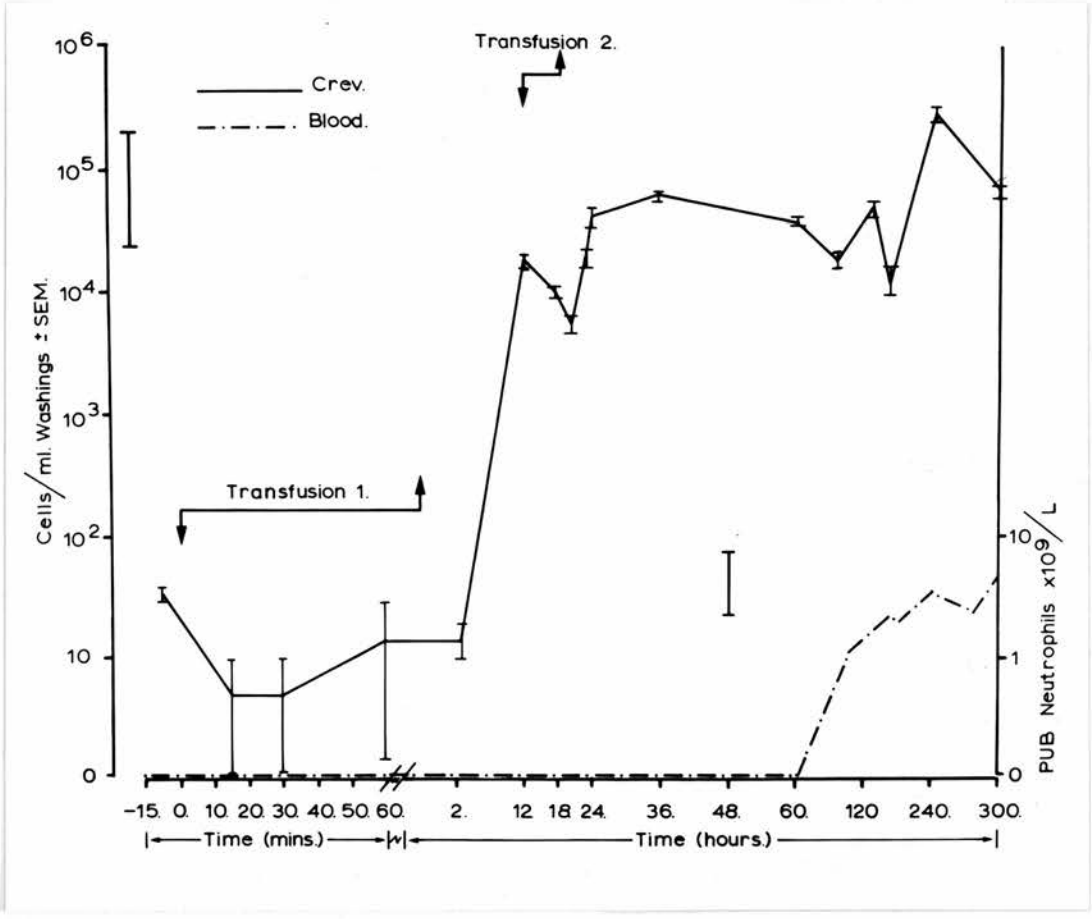

FIG. 5.7: Peripheral venous blood and crevicular neutrophil counts in a 15 year old female patient with AMØL in response to two granulocyte transfusions (Case IV). Normal values are represented by vertical bars. The sem values of the counts for crevicular cells are shown.

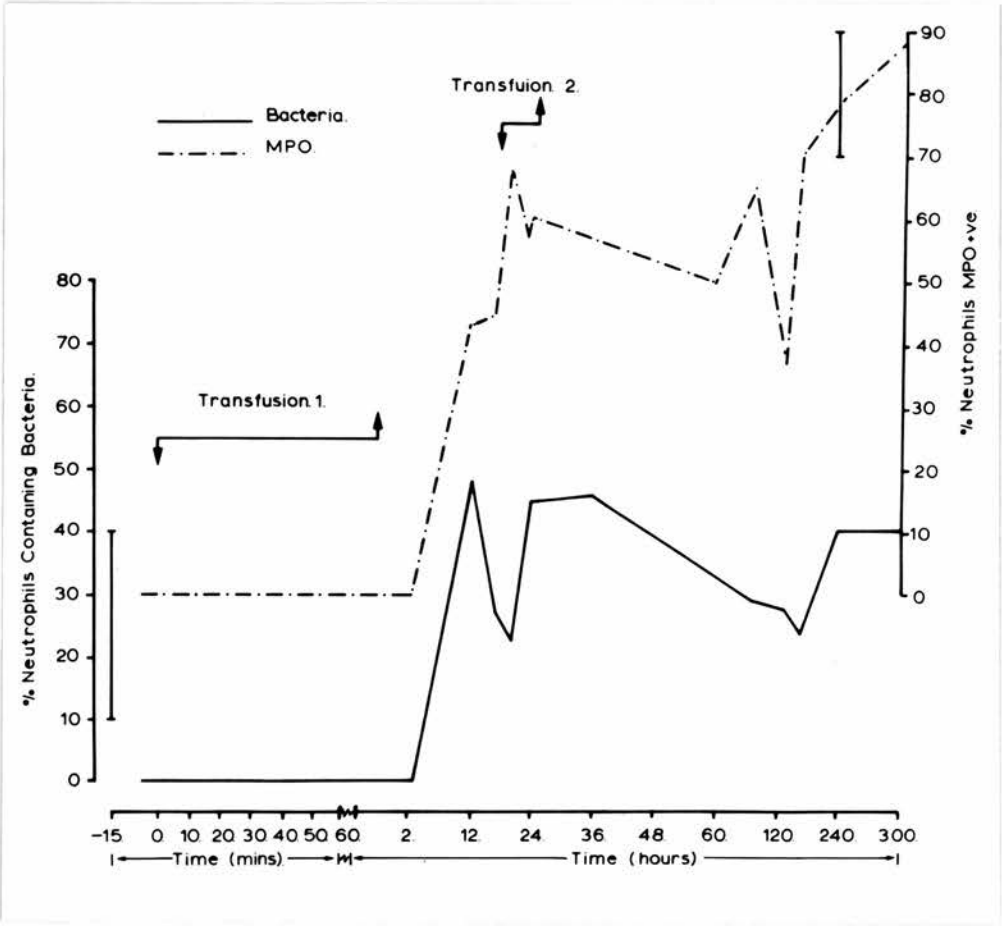

FIG. 5.8: Crevicular neutrophil MPO (cytochemical staining with benzidine) and in vivo phagocytosis in response to granulocyte transfusion (Case IV). Normal values are represented by the vertical bars.

until just before the patient died. The results are shown in the upper part of Fig. 5.9. The values were somewhat erratic but the activity fell sharply to zero on day 57 and did not recover. This was close to the time at which the PVB neutrophil count started to fall (Fig. 5.6). An NBT test performed on the crevicular cells 132 hours after the start of transfusion, by which time the cell count was high, possibly with donor and host cells, showed 43.1% of the neutrophils to be positive, i.e. a normal value. On day 61, early in the subsequent decline, the NBT response was 11.6% positive cells.

Case V: Acute Myelomonocytic Leukaemia

An acutely ill, 25 year old married woman (JMcC) was admitted, having been tired and generally unwell for approximately one month. She reported bruising easily and had "lumps" in her neck. General haematological investigation led to a diagnosis of acute myelomonocytic leukaemia (AMMøL) in which the bone marrow was infiltrated by equal numbers of each blast type. Unlike the previous two cases of acute leukaemia, the peripheral blood picture on presentation was a WBC count of 150.7 x 10^9 cells/L, with a differential count of 4% neutrophils, 1% lymphocytes and 95% blast cells. The absolute mature

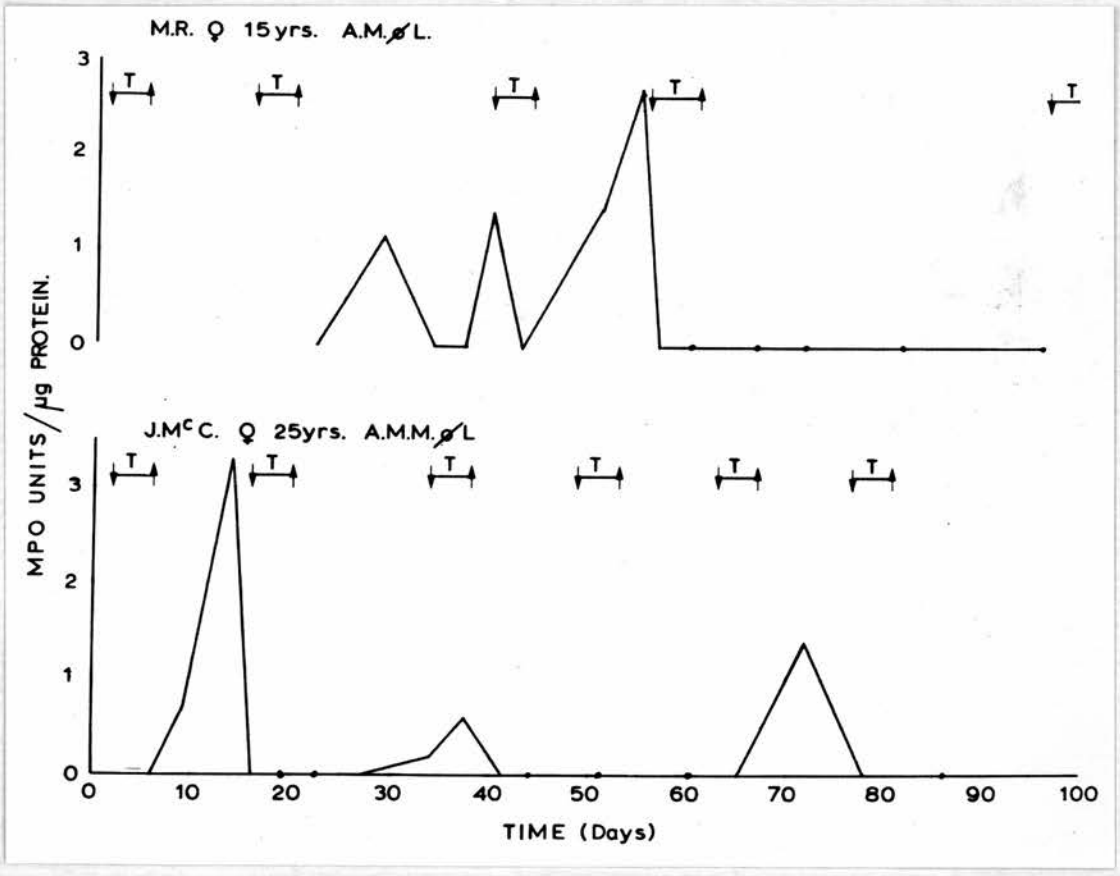

Fig. 5.9: Crevicular neutrophil MPO activity in two female patients with acute leukaemia (Cases IV and V). Bars marked T indicate pulses of chemotherapy.

neutrophil count was 6.02, i.e. within the normal range. A crevicular neutrophil NBT test demonstrated 28.6% positive cells. Unfortunately, six courses of combined chemotherapy were unable to successfully control the marrow proliferation, so that blast cells were never cleared from the peripheral blood, and the thrombocytopenia and anaemia produced significant clinical problems. She died from a cerebral haemorrhage three months after diagnosis.

Figure 5.10 shows the crevicular neutrophil counts monitored throughout, compared with the peripheral blood neutrophil counts. In general, the crevicular counts paralleled those of the blood. The simultaneously performed functional assays of *in vivo* phagocytosis and cytochemical MPO are shown in Fig. 5.11. Phagocytosis started to decline noticeably before the cell counts in Fig. 5.10. Although there were similar trends between the two functional measurements, they were less closely matched than those in the previous patient during transfusion.

The crevicular neutrophil MPO activity was determined on 17 occasions, and the values are shown with those from the previous girl in the lower part of Fig. 5.9. Twelve of the 17 values were zero and once again it is not possible to distinguish the effects of the disease from those of the chemotherapy.

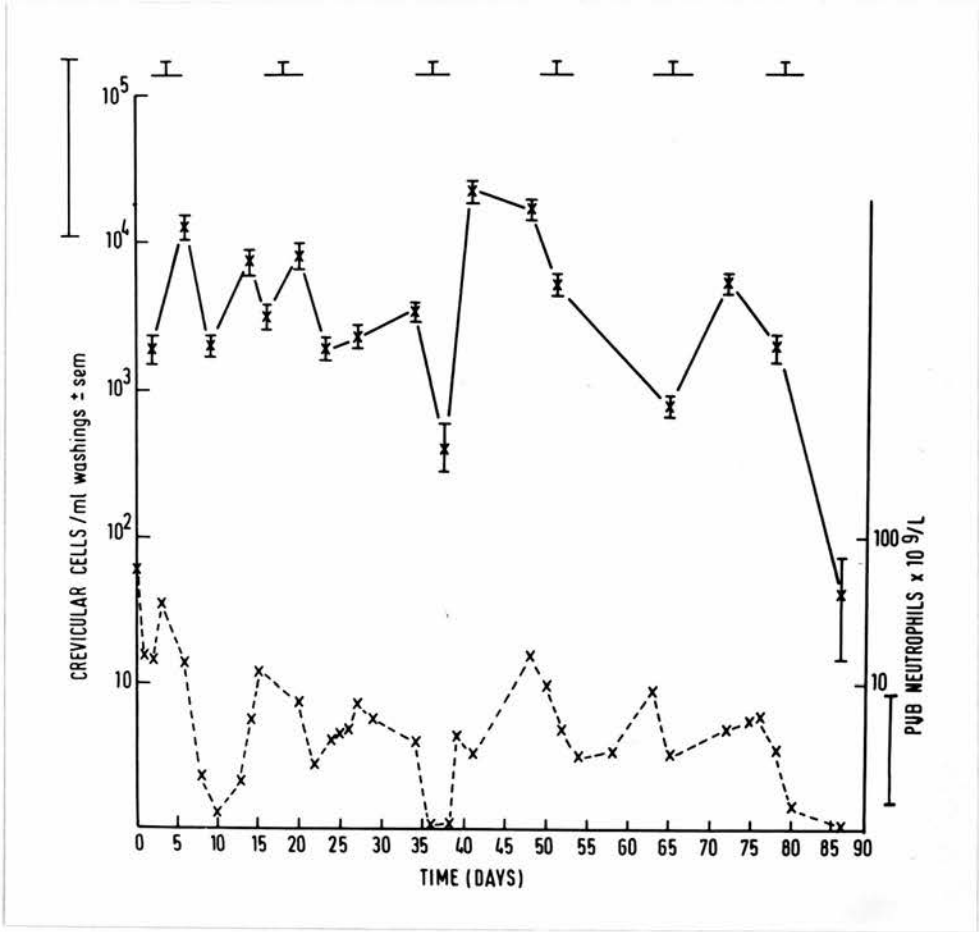

FIG. 5.10: Peripheral venous blood (----) and crevicular (———) neutrophil counts in a 25 year old female patient with AMMøL (Case V). The bars marked T represent periods of chemotherapy. Vertical bars indicate normal values.

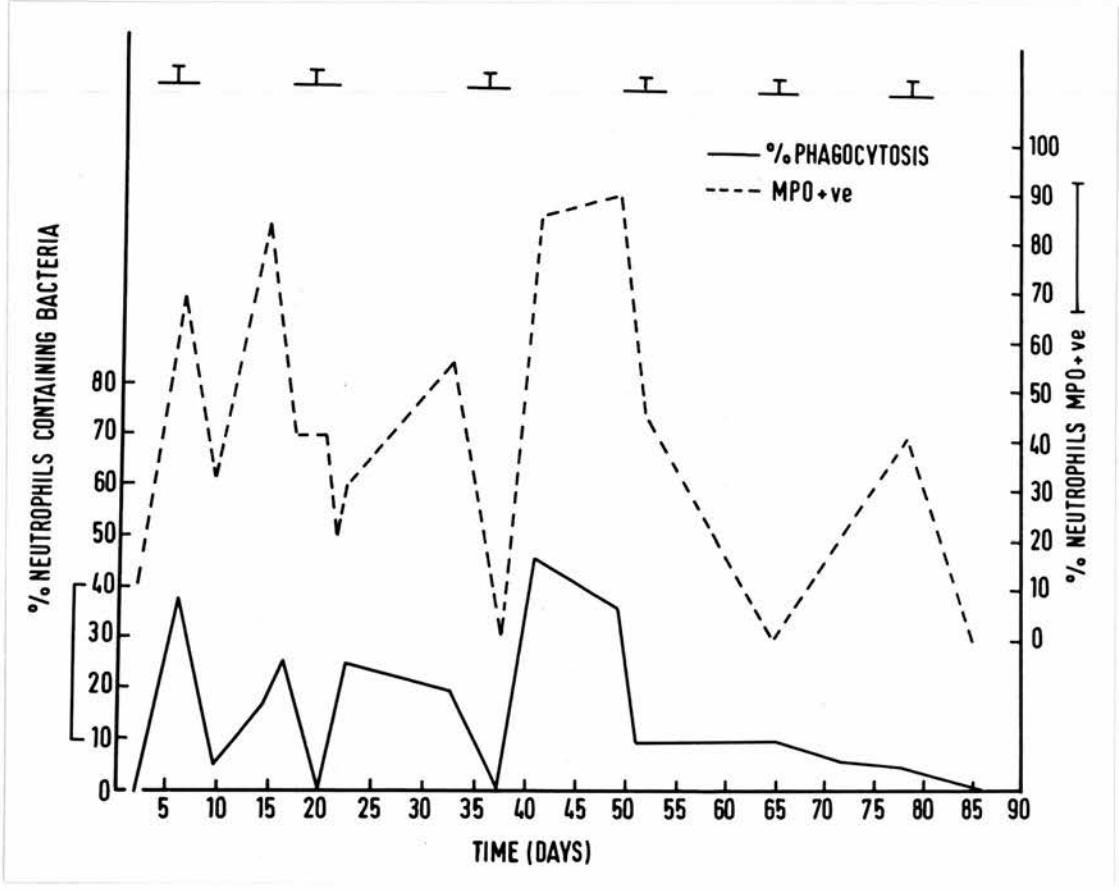

FIG. 5.11: In vivo phagocytosis and cytochemical MPO determination in crevicular neutrophils of a 25 year old female patient with AMMøL (Case V). Periods of chemotherapy are marked as T. Normal values are represented by vertical bars.

Conclusions

It would not be appropriate to generalise as to the nature and function of crevicular cells in leukaemia from the study of five cases, but several points have emerged. The individuality of disease in each patient became increasingly apparent. The first and most obvious observation was that, as for the neutropenic patients, it was easily possible to collect and study crevicular cells in patients with leukaemia. Perhaps the most important practical point was that the method of cell collection is non-invasive and therefore atraumatic. This may be a critical consideration in terms of research being conducted on extremely ill patients. Even so, it was considered inappropriate to disturb some patients and this produced some incomplete studies. Problems in interpretation were created by the difficulty in determining which factors were responsible for the observed changes. At any one time the patients were leukaemic, immunocompromised, or infected and were taking a combination of cytotoxic and antimicrobial drugs. This aspect will require to be studied further.

Results from both CML and the acute leukaemia patients studied suggested that crevicular cell functions, particularly MPO activity, were sensitive to the disease and/or cytotoxic therapy. It is tempting to suggest

that crevicular studies indicate the seriousness of a patient's condition (cf. MPO in DN and MR, and *in vivo* phagocytosis in patient JMcC).

The results for crevicular cell studies showed well established patterns when compared with blood. For example, the low blood NAP values typical of CML were shown in the crevicular cells of patient DN. Also, in the same patient, initially low cytochemical MPO measurements became normal as marrow function improved.

Another important feature was the indication that the circulating cells migrate rapidly to sites of bacterial challenge such as the gingival crevice. In patient FO there was a persistent neutropenia, and yet sufficient cells were always present in the gingival crevices to permit an estimation of MPO activity. These cells were also endowed normally with MPO even if activity was absent. This phenomenon was still more pronounced with the granulocyte transfusion studies. Although the donor cells were not labelled, it seemed that they migrated immediately in the neutropenic 'vacuum' that had been created. Thus cells were not evident in peripheral blood until 60 hours after the start of the first transfusion, which was 48 hours after the first significant increase in crevicular neutrophil numbers.

A possible relationship between cell numbers in blood and in the crevice was raised with respect to neutropenia. The studies on MR and JMcC suggested that there may be such a correlation in leukaemic individuals during therapy, especially when the peripheral blood circulation contains normal numbers of neutrophils (seen to some extent in JMcC). The relationship may not hold when the equilibrium between the marginating and mainstream circulating populations in the vessels alters. Preference may be given to the marginating cells (including those destined for the gingival crevice), with the result that none appear when blood is taken by venepuncture. Passage to the tissues is, of course, potentially the predetermined fate of all functional neutrophils. Radio-labelling of donor cells would be the best way to calculate the proportion of these cells that migrate to a given site such as the gingival crevice.

A normal NBT response was not seen in crevicular cells from the three patients with acute leukaemia except at the end of the transfusion period in MR. In the chronic leukaemias, normally segmented neutrophils were always present so that the same abnormality did not occur. Nitroblue tetrazolium reduction was normal in both CML patients but these isolated NBT tests were performed at different stages of different diseases under different treatment regimes.

A final point was the notable absence of leukaemic blast cells in the crevicular washings. This is perhaps logically explained in that presumably only fully functional cells are able to respond to a chemotactic stimulus and to migrate through blood vessels and connective tissue onto the mucosal surface. By the same argument the transfused cells were functional since they all apparently marginated and migrated immediately from the circulation, and subsequently the patient's clinical condition improved.

CHRONIC GRANULOMATOUS DISEASE

Introduction

Chronic granulomatous disease (CGD) is a rare functional disorder of phagocytic cells, which has been extensively investigated both for the insight into normal neutrophil physiology and because of the link between the disease and a molecular abnormality. In 1977, Johnston and Newman stated that 162 cases of CGD had been reported over the previous ten years and one year later Klebanoff and Clark (1978: 644-645) reviewed evidence from 226 cases described in the literature. Such has been the interest in this rare disease that several extensive and comprehensive reviews have been published (Klebanoff and Clark, 1978: 641-709; Gallin and Fauci, 1983; Hitzig and Seger, 1983).

The disease was first clearly described by a group of workers in Minneapolis (Berendes et al., 1957; Bridges et al., 1959). Severe recurrent infections in males were associated with generalised granulomatous lesions. Frequently these infections proved fatal, during childhood. Hypergammaglobulinaemia and normal phagocytosis were recorded and the four patients in the initial study died in childhood. Holmes et al. (1966)

proceeded to show that bacteria survived for prolonged periods within neutrophil phagolysosomes and that the essential defect was in oxidative metabolism (Holmes et al., 1967). This latter study demonstrated that phagocytosis was associated with an increased oxygen uptake of only 36% in CGD neutrophils as compared to 520% in normal cells. There was a concomitant reduction in HMPS activity and hydrogen peroxide formation. Subsequently many workers have investigated in detail neutrophils from patients with CGD. In general, the production, morphology, phagocytosis and chemotactic response of neutrophils have been reported as normal (reviewed by Klebanoff and Clark, 1978: 656-663). All studies have confirmed the Minnesota reports that the defect is in microbicidal activity and related to a deficient metabolic burst. Thus oxygen consumption, hydrogen peroxide production, superoxide anion formation, chemiluminescence, NBT reduction, HMPS activity and iodination are all impaired (Klebanoff and Clark, 1978: 659-663).

The failure of CGD neutrophils to reduce NBT was first described by Baehner and Nathan in 1967. A negative result from a stimulated test has now become a definitive diagnostic test. Such has been the confidence in this test that it is used for prenatal diagnosis of CGD (Newburger et al., 1979; Borregaard et al., 1982). However, there is one report of positive

NBT reduction in a boy with the classical X-linked form of CGD (Borregaard, Cross et al., 1983). An absence of NBT positivity would be expected if superoxide anion formation is deficient. Lack of superoxide production in CGD neutrophils has indeed been demonstrated by several authors (Curnutte et al., 1974; Weening et al., 1975; Rosen and Klebanoff, 1976).

Over the past few years the focus of interest has been the membrane oxidase (discussed in chapter 3) and in at least some forms of CGD this enzyme is either totally absent, or present but inactive (reviewed by Babior, 1983). Moreover, Segal (1983) has reviewed the evidence for involvement of the b_{-245} cytochrome in this system and has emphasised that the different genetic variants of the disease are associated with different oxidase defects. For example, Segal and Jones (1979) suggested that in classical X-linked CGD the cytochrome is missing, and subsequently confirmed this in a large multicentre study (Segal, Cross et al., 1983). In one of the recent reviews, Hitzig and Seger (1983) have detailed the six molecular defects associated with the X-linked form of CGD and a further three associated with the autosomal recessive form.

The clinical manifestation of the molecular lesion is a variably increased susceptibility to infection. However, it has been recognised for some years that the

organisms involved generally fall into one category, that is they do not produce hydrogen peroxide or destroy any formed with endogenous catalase (Klebanoff and Clark, 1978: 670). The most frequently encountered pathogens are Staph. aureus, Gram negative bacilli, Candida albicans and Aspergillus species (Klebanoff and Clark, 1978: 654-655; Regelmann et al., 1983; Weening et al., 1983). These organisms are phagocytosed and may remain viable for protracted periods within the cell. Klebanoff and White (1969) suggested that bacteria which do manufacture large quantities of hydrogen peroxide, e.g. streptococci and lactobacilli, can replace that missing from the neutrophils. Their work was confirmed by others (Mandell and Hook, 1969). The MPO-mediated oxidative killing system can then operate normally utilising hydrogen peroxide derived from the bacteria (Stossel et al., 1972). However, there is one interesting study which reported normal killing of Staph. aureus by CGD neutrophils in vitro in which it was assumed that much of the bactericidal capacity was due to non-oxidative pathways (Segal et al., 1982).

Monocyte function is also decreased in most patients with CGD (Klebanoff and Clark, 1978: 667) to an extent which depends upon the basic defect (Donowitz and Mandell, 1983).

Two principal modes of inheritance have been recognised. The more usual X-linked form which only affects males, and an autosomal recessive form in which female cases also occur (Klebanoff and Clark, 1978: 642). The inheritance patterns have recently been reviewed (Mills and Quie, 1983), but the predominance of the X-linked disease is illustrated by the review published by Klebanoff and Clark (1978: 644-645) in which 202 were male and 24 female. In the female carriers there are two populations of neutrophils resulting from the inactivation of one of the X chromosomes of each cell - so-called Lyonization. No abnormalities are detected in the fathers or unaffected male siblings (Hitzig and Seger, 1983). According to the Lyon hypothesis (Lyon, 1972) carriers show no intermediate stages of activity between the two cell populations, i.e. one group is entirely normal and the other entirely abnormal. By chance the relative proportions of the two cell types within one individual may vary depending on the inactivation of the normal or the defective X chromosome (Mills and Quie, 1983). Neutrophil activity overall should be intermediate in the female carriers of CGD and this was again shown by the Minnesota group (Windhorst et al., 1968) using an NBT test and subsequently confirmed by others (Ochs and Igo, 1973; Hitzig and Seger, 1983). Microbicidal activity (Suzuki et al., 1971) and chemiluminescence (Rosen and Klebanoff, 1976) measurements

have also been intermediate between control values and CGD patients. The studies of Segal's group mentioned above have demonstrated an absence of cytochrome b_{-245} in CGD neutrophils and it was further shown that this deficiency was also partial in the carriers (Segal, Cross et al., 1983).

Over the past 20 years the mainstay of management in CGD has been antibiotic therapy to control and treat infection. Since one of the principal problems in CGD patients is the survival of organisms within phagocytes, recently increased success in treatment has been attributable to the use of antibiotics which gain access to the interior of the cell, for example, rifampicin. This subject has been well reviewed (Klebanoff and Clark, 1978: 688-689; Johnston, 1983). The combination of sulfamethoxazole and trimethoprim has also been used to good effect (Gmünder and Seger, 1981; Seger et al., 1981; Weening et al., 1983), due probably to the high drug concentrations obtained within the neutrophils. Seger et al. (1981) showed that the cells concentrate sulfamethoxazole three-fold and trimethoprim fourteen-fold. Granulocyte transfusions have been given to CGD patients during severe infection but with variable results (reviewed by Johnston, 1983). Buescher and Gallin (1982) have proposed that monocyte transfusion would be more appropriate, since these cells persist longer in the infected tissues.

The prognosis for patients with CGD has been improved by the more judicious use of antimicrobial drugs, coupled with an increased understanding of the cellular lesion. There is, however, no room for complacency since the disease can still prove fatal particularly if highly pathogenic organisms are involved (Seger et al., 1982).

Crevicular neutrophil studies were conducted in one boy with diagnosed CGD, and as part of an investigation into carrier status in the female members of another family, in which two boys with X-linked CGD had died.

Case I: Chronic Granulomatous Disease

A young boy (MS) first presented with severe infection problems at the age of 6 months. Total leucocyte and absolute neutrophil counts were normal and no immunological abnormalities were revealed. At one year of age, a neutrophil NBT test gave a low result, at the time of an acute admission with an abdominal abscess. Cytochemical MPO and in vitro candida killing were normal. The NBT test results for MS and his immediate family are shown in Table 5.7. These tests were performed by a service microbiology laboratory. Not only did these data illustrate the patient's defect but suggested carrier status in the mother.

MS has continued to suffer from repeated ear and upper respiratory tract infections which have been successfully controlled with cotrimoxazole. His mother has been confirmed as a carrier. A young brother suffers from an excess of infections but a diagnosis of CGD has not yet been confirmed.

At the age of 6 years, crevicular and PVB neutrophil studies were performed and the results are presented in Table 5.8. The state of the teeth and oral soft tissues was extremely good. Twenty deciduous teeth were present with no caries and no restorations. Cell counts in both crevice and blood were normal as was *in vivo* phagocytosis in the crevicular neutrophils. Reduction of NBT was totally absent in blood neutrophils but seen in 8 out of 1,000 crevicular cells scored. The only other point of note was that the blood cells possessed extremely well-formed and prominent granules, as seen on the cytocentrifuge preparations.

TABLE 5.7:

PERIPHERAL VENOUS BLOOD NEUTROPHIL NITROBLUE TETRAZOLIUM REDUCTION (PERCENTAGE POSITIVE CELLS) IN A FAMILY WITH CHRONIC GRANULOMATOUS DISEASE

Subject	Unstimulated	Stimulated
MS	0	0
Mother	3	15
Father	5	29
Control	4	37

TABLE 5.8:

PERIPHERAL VENOUS BLOOD AND CREVICULAR NEUTROPHILS IN CHRONIC GRANULOMATOUS DISEASE: MS AGED 6 YEARS

Source	Neutrophil No. x 10^9/L	Differential %	*In vivo* Phagocytosis %	NBT Reduction (% +ve cells)	NBT Stimulated
Blood	3.72	49	-	0*	0*
Crevice	0.0094	100	18	0.8*	-

* Abnormally low values

Case II: Carrier Status in X-Linked Chronic Granulomatous Disease

In a family in which there were six children, two girls and four boys, two of the boys had died at aged 10 months and 7 years respectively, following histories of severe, recurrent infections. Neither parent had suffered significantly from infections, nor had any of the other four children. Neutrophil function tests performed on the 7 year old boy led to a diagnosis of classical X-linked CGD.

At the age of 22 years the elder daughter decided to marry, and since her mother was an obligate carrier, she had a 1:2 risk of also carrying the disease. Crevicular and blood neutrophil NBT tests were performed and the results are presented in Table 5.9.

TABLE 5.9:
PERIPHERAL VENOUS BLOOD AND CREVICULAR NEUTROPHIL NITROBLUE TETRAZOLIUM REDUCTION (PERCENTAGE POSITIVE CELLS) IN A POSSIBLE CARRIER OF X-LINKED CHRONIC GRANULOMATOUS DISEASE

Source	Unstimulated	Stimulated
Blood	2.9 (5.1)	16.7 (19.6)
Crevice	33.3 (34.1)	-

Figures within brackets = control

The results did not suggest any significant deficiency in NBT reduction.

Having married, the patient went to live in Holland, and by arrangement specific neutrophil oxidative function was investigated. Oxygen consumption, hydrogen peroxide formation and NBT reduction (all blood neutrophils) were normal. The conclusion was therefore that she was not a carrier. (She has since had a healthy daughter.)

The younger daughter was 15 years old when first seen for crevicular cell studies. Crevicular neutrophils from her father, mother (identified carrier) and an unrelated control subject were tested simultaneously. The mean results are depicted in Fig. 5.12. The values for the father and control subject were normal, but those for the girl and her mother were approximately 50% of that for the control subject and 60% that of the father. The indication was therefore that this daughter is a a carrier, subject to confirmation.

Conclusions

The molecular bases of the microbicidal defect in CGD have now been largely elucidated and in the course of this work much has been learned about the biology of neutrophils. In fact, Segal (1981) suggested that CGD

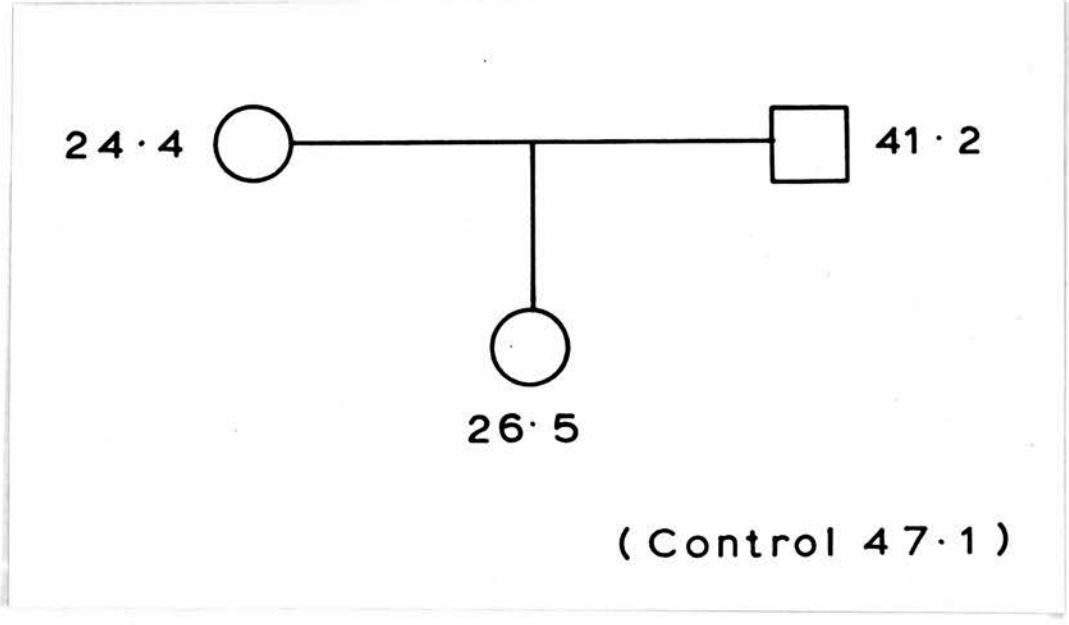

FIG. 5.12: Crevicular neutrophil NBT reduction values (percentage positive cells) in three members of a family under investigation for carrier status in CGD (Case II). The value for a healthy control subject is also shown.

patients would be ideally suited to genetic engineering, should this ever become practicable.

The crevicular neutrophil data presented from two investigations were extremely limited, but the nature of the data would seem to justify discussion. In the case of the boy with diagnosed CGD in whom the history had been classical and the mode of inheritance confirmed, the crevicular neutrophils reflected the inactivity of those in the peripheral blood. Unlike the blood cell preparations where the total negative response was striking, a very small number of crevicular cells was positive (8 in 1,000). In comparison to normal test results, this value is insignificant, and it is possible that a technical artefact was responsible. It is hoped that further studies in this family, particularly of the mother and younger brother, will be possible.

Oral lesions are not a prominent feature of CGD, and the limited literature has been reviewed recently (Donowitz and Mandell, 1983). In most cases the only feature of note is minor oral ulceration although with an incidence not above that for the general population (Scully, 1981; Donowitz and Mandell, 1983). There is one recent report of a case in which ulceration and gingivitis were severe (Allan and Straton, 1983). The oral condition of MS was excellent and, having no gingivitis, caries or restorations, he was far better than the norm for his age. This may have been due to his

mother's conscientious approach. Another factor is that the organism principally responsible for human dental caries is Streptococcus mutans, a hydrogen peroxide-producing bacterium. It should therefore be possible for CGD neutrophils to successfully kill these bacteria. This is clearly in contrast to the situation in neutropenia where problems are caused by there being insufficient phagocytes. It is not known whether this patient will develop aggressive periodontal disease in later life, but available evidence suggests that this should not be the case (Donowitz and Mandell, 1983).

In the family study the typical inheritance pattern of X-linked CGD was seen, both in terms of the disease transmission and its carriage. Two sons had died, one in infancy, and the infection history and pathology were classical. The other two boys had no history of infection problems and were shown to be completely free of any immunological disorders. The elder daughter had normal neutrophil function as determined by NBT reduction both in the crevice and blood, and this was verified for blood neutrophils by more extensive tests conducted in another laboratory (by Drs Roos and Weening, Amsterdam and Professor van Furth, Leiden). The chance of her being a carrier (1:2 theoretically) is thus reduced. The younger daughter will require to be investigated thoroughly to exclude or confirm her being a carrier.

However, since the crevicular cell studies for the first daughter had apparently reflected the blood picture, then in this girl the same relationship may apply. Crevicular neutrophil NBT reduction was similar to that in her mother. Such a result would be consistent with those from blood neutrophil studies in carriers (Repine et al., 1975).

Crevicular neutrophil studies may therefore prove useful both in terms of a research application to CGD and possibly as an adjunct to clinical diagnosis.

DISCUSSION

The limited studies that have been described, conducted on patients with a variety of neutrophil disorders, have provided data from which tentative conclusions have been drawn and possibilities for further research suggested.

The importance of neutrophils as a primary barrier against infection in host defence has been underlined by the deficiency states. In severe neutropenia, whether congenital or associated with leukaemia, infection is likely to occur due to the quantitative defect of phagocytes. In the dysfunctional state of CGD, systemic infection is also seen but generally less severely than in neutropenia and leukaemia and involving a narrower spectrum of pathogens. The oral cavity may also be a clinical marker of neutropenic states, but not CGD. In neutropenia the typical oral ulceration and severe gingivitis were present in one patient (JS), who also presented with early destructive periodontitis which is very rarely seen in healthy children of the same age. His father had lost all his teeth in early adult life due to severe periodontal disease.

Clinical problems in mucosal sites seem to be associated mainly with a deficiency in cell numbers, rather than of function. In the leukaemic patients

only segmented neutrophils appeared in the exudate washings. No blast cells were seen, and this is consistent with the assumption that only normal functional cells would be capable of responding to a chemotactic stimulus. An investigation into the relationship between the cells present and the clinical state of the tissues in the patients could provide useful information concerning the role of neutrophils in periodontal disease. One study has reported finding leukaemic blast cells in the gingival crevicular washings of children with acute lymphoblastic leukaemia (Sonis and Sonis, 1981) but several aspects of the methodology were open to criticism. If immature and blast forms are incapable of migrating from the circulation, it may leave a relatively high concentration of these cells in the mainstream circulation, and not in a normal equilibrium with the marginating population. Results of differential counts from peripheral blood may then inaccurately reflect the true proportions of the cell types leaving the bone marrow. Marrow biopsy would indicate the actual state of cell production and PVB and crevice studies would indicate the kinetics of migration.

The concept of monitoring marrow activity through cell migration into the oral cavity was discussed nearly 50 years ago, when Allen and Dickey (1937) suggested that salivary leucocyte counts were useful indicators of marrow function in CML, following irradiation therapy.

More recently, Arnold et al. (1977) counted salivary cells before and after granulocyte transfusion. As was the case in the present study, they reported the presence of transfused cells in saliva before they appeared in the peripheral blood. The passage of cells into saliva with time was not monitored, nor any functional characteristics. In the single granuolcyte transfusion monitored in this study, neutrophils appeared relatively quickly in the crevicular washings, but it is not known whether this was prior to, concomitant with or following their migration to other sites, such as the lungs. More detailed labelling studies are required but Saverymuttu et al. (1983) have stressed that in labelling studies the cell function may be significantly altered by the labelling technique itself. Nevertheless, this type of investigation could be extended to provide more information about the kinetics of migration in such patients.

During transfusion in this study, the cell counts appeared to alter markedly. Even when the number was very low prior to transfusion, exudate fluid was evident. This observation supported the findings of Kowashi et al. (1980) which showed that the cellular and fluid components of crevicular exudate are under independent control.

The present studies have also confirmed the convenience and validity of the gingival crevice as a

site for study of cell migration and function. Samples are available, completely without trauma or invasion, of particular importance in an immunocompromised patient.

In the CGD studies both in the patient and carriers, the crevicular cell activity accurately reflected that in the blood neutrophils. In the case of the leukaemic subjects, a relationship was less apparent and should be further investigated. Parameters, easily measured in crevicular cells, might show promise as predictors of relapse or remission during treatment. These isolated results hint at such a possibility. In one CML patient (Case I), crevicular neutrophil MPO activity was normal on several occasions following breast cancer therapy. In two patients, one with CML (Case II), and one with AMøL (Case IV), crevicular MPO activity ceased some considerable time before the patients died. In vivo phagocytosis also decreased latterly in the young woman with AMMøL (Case V). Cytotoxic chemotherapy appeared to depress MPO activity in the patient with AML (Case III), but in the long term study of one CML patient (Case II), the crevicular neutrophil cytochemical MPO reflected the improvement induced by the chemotherapy.

Generalisations applicable to a broad category of patients may not be possible, since cellular activity varies from the outset. In this type of investigation, sequential studies in individual patients may be

particularly appropriate. Suda et al. (1983) reported 34 out of 35 patients with AML who presented with abnormal blood neutrophil cytochemistry, and who could be classified into three groups according to the changes resulting from therapy. Patterson et al. (1982) reported one patient with AML as having abnormally high neutrophil peroxidase activity. Recent studies with blood neutrophils have suggested that disease and prognosis, to some degree, may be predicted from some functional markers. Solberg et al. (1975) showed that decreased neutrophil bactericidal function preceded the development of overt leukaemia in five patients. Bendix-Hansen and Kaspersen Nielsen (1983b) found that MPO activity had no predictive capacity, but did normalise once therapy was controlling the leukaemia. By contrast, another recent study showed that azurophilic granule enzyme activity (including MPO) fell in advance of a relapse in AML (Schofield et al., 1983). The crevicular studies mentioned above would accord with this latter finding.

The crevicular sampling procedure is also applicable when few cells are available for functional studies, as in severe neutropenia. Dale and Wolff (1971) performed studies on neutropenic patients using the skin window technique, but this is an invasive procedure. Also the initial neutrophilic infiltrate in a skin window is subsequently replaced by monocytes, a sequence which does

not occur in the gingival crevice. Freeman and King (1972c) described a blood NBT test suitable for neutropenic patients and Gordon et al. (1975) developed a test by which blood neutrophils were concentrated by cytocentrifugation, so facilitating study in neutropenia. Collecting sufficient crevicular cells for study in the neutropenic states so far encountered has presented no difficulty, with the exception of the transfusion patient (Case IV) immediately prior to treatment, when the crevicular counts were extremely low (and cells appeared to be non-existent in peripheral blood).

Summary

It has been demonstrated that collecting crevicular neutrophils for study from patients with disorders of neutrophil production and function is practicable. The procedure is atraumatic and the results suggest that further investigations should demonstrate the usefulness of this approach both in neutrophil research and clinical monitoring of disease and treatment.

CHAPTER 6

GENERAL DISCUSSION

In the context of host defence, neutrophil leucocytes provide non-specific protection against microbial invasion, and it is often implied that their function is less sophisticated than the specific mechanisms of immunity. It would be more appropriate to regard this ability to deal with a wide range of organisms as a highly successful attribute. In a wider perspective of cell biology, the neutrophil is highly developed for its intended function.

The structure of a neutrophil bears little resemblance to standard text book drawings of a 'typical cell', which show a single nucleus and the cytoplasm replete with many extra-nuclear organelles. The nucleus in a mature neutrophil is multilobed and distinctive whilst the cytoplasmic membrane structures of endoplasmic reticulum, ribosomes and mitochondria are almost entirely replaced by membrane structures of a different form. These are the lysosomes and granules, all charged with the enzymatic and biochemical means necessary to achieve the cell's defensive objectives. This development is further seen in a unique feature of phagocytic cells, that is the ability to generate considerable microbicidal activity from the progressive reduction of molecular oxygen. In the virtual absence of mitochondria the neutrophil produces energy equivalents (ATP molecules) from glycolysis. It may be that those membrane structures,

once designed for oxidative phosphorylation, have been transformed into those now reducing oxygen by a different pathway to superoxide and hydrogen peroxide as a basis for the oxygen-dependent microbicidal systems.

The importance of neutrophils to defence is also shown by the greatly increased susceptibility to infection seen in neutrophil deficiency states, particularly those affecting cell production. A recent report of ten cases of septicaemia, one fatal, in seven of which recovery was dependent on the provision of adequate granulocytes rather than the use of antimicrobial drugs, emphasises this role (Cohen et al., 1983). Equally relevant to this discussion was the identity of the organisms which caused the septicaemia. All were viridans streptococci, which are usually regarded as commensal and present universally in the oral cavity. The pathogenic status arose from the absence of the normally controlling neutrophils. The authors suggested that the portal of entry to the circulation, ironically, may have been mouth ulcers caused by cytotoxic chemotherapy. The oral cavity is therefore a site of critical importance to systemic host defence and the function of neutrophils in one part of the mouth, the gingival crevice, has been the subject of this study.

Neutrophils in the Gingival Crevice and Periodontal Disease

The presence of neutrophils in the gingival crevice has been recognised for many years but the first functional studies were reported comparatively recently (Wilton *et al.*, 1977a,b). These were limited to basic assays of phagocytosis and killing, using *Candida albicans* as the *in vitro* test organism. An interesting observation noted in one paper (Wilton *et al.*, 1977b) was the toxicity of crevicular fluid for autologous peripheral blood neutrophils and yet a subsequent report described the strong opsonic activity towards oral organisms (Wilton *et al.*, 1983), so illustrating the complexity of possible interactions in an inflammatory exudate. The decreased phagocytic activity of crevicular as compared to blood neutrophils (Wilton *et al.*, 1977a; Scully, 1982) may be the result of an intrinsic cellular defect or could be an acquired deficiency produced by some component of the fluid or associated with the physical passage of cells through connective tissues. The results of the present study have demonstrated *in vivo* phagocytosis, NBT reduction and functional MPO in crevicular neutrophils. Whilst the crevicular cells were more susceptible to the toxicity of NBT, and overall had less MPO activity than autologous blood neutrophils, their MPO activity was markedly increased by the antibiotic

cefaclor in comparison to blood cells.

The work of Scully has demonstrated the ability of oral neutrophils to phagocytose and kill Streptococcus mutans, which is clearly implicated as a causative organism in human dental caries. This activity is enhanced by the presence of specific opsonising antiserum (Scully and Lehner, 1979b). However, a problem that has hardly been addressed is the ability of crevicular neutrophils to phagocytose or kill bacteria implicated in human periodontal disease. The basic model for such an investigation has been the in vitro interaction between bacterial species isolated from dental plaque and peripheral blood neutrophils. Some of these studies have demonstrated the ability of plaque organisms to induce extracellular lysosomal enzyme release from neutrophils and thus have hypothesised mechanisms of tissue destruction (e.g. Baehni et al., 1979). Others have demonstrated the inhibitory effects (e.g. Adamu and Sperry, 1981) or cytotoxicity (Shurin et al., 1979; Taichman and Wilton, 1981; Nowotny et al., 1982) of plaque bacteria for neutrophils. While providing useful information and indicating directions for research, more information is needed concerning the ability of crevicular neutrophils to adequately control potential pathogens. The only indications of such a capability have been from electron microscopic studies (Garant, 1976; Newman, 1980; Saglie et al., 1982).

In the present study the correlation between MPO activity and gingival inflammation may indicate a causal relationship, but such a finding may result from indirect causes coincidental to other tissue changes. It requires further study. Myeloperoxidase has many roles in neutrophil function and in crevicular cells one of these must be the classical catalysis of microbicidal oxidations. The enzyme may also interact with protease inhibitors to enhance inflammation (Clark et al., 1981). Proteases (Kowashi et al., 1979) and protease inhibitors (Peterson and Marsh, 1979) have been identified in crevicular fluid.

The passage of cells through the gingival tissues has been considered disruptive but there is evidence that the transmigration of neutrophils through epithelium may cause no irreversible damage (Milks et al., 1983). However, the potentially destructive role of activated neutrophils in inflammatory disease has been a popular theme in recent years (Lanser and Saba, 1981). The oxygen-dependent systems have been strongly implicated as contributing to the damage (Halliwell, 1982; Weiss and LoBuglio, 1982). There is also a strong case for tissue damage being caused by proteolytic enzymes, particularly in lung injury (Blue and Janoff, 1978; Hunninghake and Crystal, 1983). Obviously both mechanisms may operate synergistically (Matheson et al., 1979; Clark et al., 1981; Turkall and Tsan, 1982). Also, a recent report

has proposed an interesting and plausible mechanism for joint damage in rheumatoid arthritis, in that oxygen radicals, generated from activated synovial fluid neutrophils, were shown to significantly alter lymphocyte function *in vitro* (Niwa *et al.*, 1983). Most of these models have yet to be tested or applied to human periodontal disease. In this study crevicular neutrophils have been shown to reduce NBT and to possess active MPO but this is not synonymous with their being automatically agents of destruction.

Against this increasing body of evidence for a destructive role, the clinical evidence suggests that neutrophils in the oral cavity are protective. The progressive, rapid soft tissue and alveolar bone destruction seen in patients with severe cyclical neutropenia provides convincing evidence. Another aspect of the argument has been closely examined through the rare disease of juvenile periodontitis or periodontosis (Saxén, 1980). Many reports have identified a cellular chemotactic defect in patients with juvenile periodontitis (e.g. Clark *et al.*, 1977; Genco *et al.*, 1980; Van Dyke et al., 1982), and the disease is particularly marked when the neutrophil defect is aggravated by the presence of leucotoxic bacteria in the gingival crevice (Taichman and Wilton, 1981; Ginsburg *et al.*, 1982). Juvenile periodontitis may be an extreme or exaggerated form of the condition normally seen in the majority of adults.

The conclusion must be that crevicular neutrophils are both protective and destructive (Miller et al., 1984). Seen in perspective, this should be interpreted as protective for the whole organism at the expense of localised and quantitatively minimal tissue destruction. That is, the price of systemic health may be periodontitis.

Crevicular Neutrophils as a Model in Clinical Research

Kanthack and Hardy (1894) were the first workers to propose the investigation of phagocytic cells in inflammatory exudates, but such work did not begin seriously in human studies until the introduction of the skin window by Rebuck and Crowley (1955). Neutrophils exist in secretions such as colostrum and human milk, where they are functional (Ho and Lawton, 1978; Pickering et al., 1980), but a factor contributing to the relative lack of studies on secretory and exudate cells must be their inaccessibility. The gingival crevice therefore provides a site of easy access with none of the organisational difficulties of, for example, collecting cells from uterine cervix washings.

This study has demonstrated that tests of crevicular cell function may provide an additional approach to the investigation of a patient's ability to deal with microbial invasion. The correlation between NBT

reduction values for blood and crevicular neutrophils should be further investigated, since a predictive assay of host defence potential could prove most useful. Similarly, the sensitivity of crevicular neutrophil MPO to antimicrobial and cytotoxic drugs, and the ease with which cells can be harvested atraumatically, present the possibility of repeated monitoring of disease and treatment in severely ill patients, e.g. with leukaemia and neutropenia. However, the characteristics of the MPO must be more closely defined, particularly in the light of recent work which has demonstrated the influence of fluctuating hormone levels on salivary LPO (Cockle and Harkness, 1983), and the evidence for the existence of several MPO iso-enzymes (Kinkade et al., 1983).

Several groups of workers have described subsets of neutrophils in peripheral blood, identified by a variety of techniques. Crevicular cell studies may help to resolve this intriguing possibility.

Finally, in this respect, the detection of carrier status in CGD by such a simple technique could prove particularly useful in children. The minimal age of cooperation for the crevicular rinsing technique appears to be about 2½ years, by which time the deciduous dentition is established, so providing many sampling sites.

Conclusion

A century ago Metchnikoff emphasised the importance of considering both humoral and cellular components of immunity as a whole. Despite this, experiments are generally designed to study limited functions. The principle of Metchnikoff's approach must be considered in order to properly elucidate the protective and destructive aspects of neutrophils in the gingival crevice, especially as these relate to human periodontal disease. Such studies will also provide information about neutrophil function in sites of confrontation between host and microorganisms.

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APPENDICES

APPENDIX 1

COMBINED METHOD FOR NON-SPECIFIC AND CHLOROACETATE ESTERASES

REAGENTS

1. Fixative

Na_2HPO_4	100 mg
KH_2PO_4	500 mg
Acetone	225 ml
Formalin (Formaldehyde solution)	125 ml
Distilled water (Store at 4^0C)	150 ml

2. Pararosanalin solution

   Dissolve 1 g of pararosanalin hydrochloride in 20 ml of distilled water + 5 ml conc. HCl.
   Warm gently, cool and filter.
   Store at room temperature.

3. 0.066 Molar phosphate buffer, pH 7.4 (M/15) (store at 4^0C)

KH_2PO_4	0.87 g
Na_2HPO_4	3.84 g
Distilled water	up to 500 ml

4. 0.066 Molar phosphate buffer, pH 6.3 (M/15) (Store at 4^0C)

KH_2PO_4	3.5 g
Na_2HPO_4	1.1 g
Distilled water	up to 500 ml

5. Ethylene glycol monomethyl ether

6. N-N dimethyl formamide

7. Prepare the following reagents in glass, code A-D.

WEIGH:
- A- 80 mg Sodium nitrite
- B- 10 mg AS-D chloroacetate (deep freeze) in bijou
- C- 60 mg α-naphthyl butyrate (deep freeze) in bijou. (This is a liquid.)
- D- 20 mg Fast Blue BB (deep freeze)

METHOD:

1. Fix smears in fixative for 30 sec at 4-10^{0}C. Wash in water 3 times. Allow to dry (10-30 min at room temperature).
2. To Bijou C add 3 ml ethylene glycol monomethyl ether.
3. In a Coplin jar place:

M/15 phosphate buffer, pH 6.3	38 ml
Solution C	2 ml

4. To Tube A add 2 ml distilled water, mix to dissolve, then add 2 ml pararosanilin solution - mix - after 1 min add 0.5 ml to Coplin jar.
5. Stain smears for 45 min in this solution at room temperature. Wash in water 3 times.
6. To Tube B add 5 ml of N-N dimethyl formamide in glass.
7. In a Coplin jar mix:-

M/15 phosphate buffer, pH 7.4	38 ml
Solution B	2 ml
Contents of Tube D	

8. Stain slides for 20 min (longer if required at room temperature). Wash well.
9. Counterstain in 2% methyl green for 10 min.

RESULTS

α-naphthyl butyrate esterase activity (as indicated by red-brown granules) is as follows:-

STRONG	-	monocytes only
WEAK or NEGATIVE	-	myeloblasts, plasma cells, lymphocytes, granulocytes, megakaryocytes

Chloroacetate esterase activity (as indicated by blue granules) is strong in the granulocyte series.

APPENDIX 2

NEUTROPHIL ALKALINE PHOSPHATASE (NAP)

(Based on method of Kaplow, 1963)

REAGENTS

1. Fixative (10% formalin in absolute methanol)
2. Naphthol AS-MX phosphate concentrate
3. Fast Blue RR
4. 2% neutral red
5. 1% aqueous light green

METHOD

N.B.: If EDTA anticoagulated blood used, prepare film within 30 mins of collection.

1. Fix slides for 30 sec.
2. Brief rinse in distilled water.
3. Add 2 ml naphthol phosphate concentrate to 48 ml distilled water. To this, add 1 capsule Fast Blue RR. Mix. (Contents do not dissolve completely.)
4. Place in a clean Coplin jar containing slides. Leave for 2 h.
5. Wash under tap for 10 min.
6. 1st counterstain: 1% aqueous light green, 3 min. (This should be done at 4^0C.)
7. Brief rinse to remove all excess stain.
8. 2nd counterstain: 2% neutral red, 10 min.
9. Rinse under tap. Allow to dry.

ACTION

This stain works on a diazo-dye coupling technique, the alkaline phosphatase activity giving rise to colour of varying intensity in the granules of mature neutrophils.

RESULTS

Normal value is approximately 40-150.

The NAP level is raised in conditions of:

a) infection

b) pregnancy

c) contraceptive pill

d) polycythaemia vera

It is reduced in chronic myeloid leukaemia.

APPENDIX 3

NEUTROPHIL MYELOPEROXIDASE

(Benzidine Method; Kaplow, 1965)

NB: It is advisable to wear protective gloves when handling these reagents.

REAGENTS

FIXATIVE - 10 ml of 40% formalin in 90 ml absolute ethanol

STAIN -	30% Ethanol	100 ml
	Benzidine dihydrochloride	0.3 g
	$ZnSO_4.7H_2O$, 0.132M (3.8% w/v)	1.0 ml
	$NaC_2H_3O_2.3H_2O$	1.0 g
	3% H_2O_2	0.7 ml
	NaOH, 0.1N	1.5 ml
	Safranin	0.2 g

The reagents must be added in the above order, mixing well. There may be some residue. The final pH should be 6.0 ± 0.05. The solution should be filtered and stored at room temperature, for up to six months.

METHOD -

1. Fix freshly prepared films for 60 sec at room temperature.
2. Rinse in tap water for 30 sec.
3. Immerse in staining solution for 30 sec at room temperature.
4. Rinse in tap water.
5. Counterstain with 1% safranin, haematoxylin or Giemsa.
6. Rinse in tap water and air dry.

RESULT -

Peroxidase-positive, azurophilic granules stain darkly.

APPENDIX 4

NEUTROPHIL MYELOPEROXIDASE

(3-Amino-9-ethylcarbazole Method; Kaplow, 1975)

REAGENTS

FIXATIVE -

40% Formalin		25 ml
Acetone		45 ml
Na_2HPO_4	20 mg	in 30 ml water
KH_2PO_4	100 mg	

Final pH - 6.6

STAIN -

Acetic acid, 0.02M, adjusted to pH 5.0-5.2 with 1N NaOH	50 ml
Dimethylsulphoxide	6 ml
3-Amino-9-ethylcarbazole	10 mg
0.3% H_2O_2	0.4 ml

Final pH - 5.5; filter solution before use.

METHOD -

1. Fix freshly prepared films for 15 sec at room temperature.
2. Rinse in distilled water.
3. Immerse film in stain for 2-3 min at room temperature.
4. Wash in tap water.
5. Counterstain with haematoxylin for 10 min.
6. Wash in tap water and air dry.

RESULT -

Peroxidase-positive, azurophilic granules stain rusty brown. Nuclei stain pale blue.

APPENDIX 5

SPECTROPHOTOMETRIC ASSAY OF MYELOPEROXIDASE

(Method of Klebanoff, 1965)

SPECIAL REAGENTS

1. Phosphate buffer 0.1M, pH 6.0. Dissolve 1.40 g $NaH_2PO_4.2H_2O$ and 0.28 g $Na_2HPO_4.2H_2O$ in 100 ml distilled water. Check pH.
2. Hydrogen peroxide 0.01M. Add 0.1 ml 30% H_2O_2 to 88 ml distilled water. Prepare freshly for use.
3. Orthodianisidine 0.2M. Dissolve 0.0488 g in 10 ml methanol. Prepare freshly for use.

ASSAY

1. Set the spectrophotometer to record at 460 nm.
2. Set thermostat of water bath to 25^0C.
3. Add 2.65 ml phosphate buffer and 0.3 ml H_2O_2 solution to a 3 cm cell.
4. To initiate reaction, add 0.05 ml orthodianisidine solution, followed immediately by 0.05 ml neutrophil preparation.
5. Mix well and follow the increase in optical density at 460 nm for 5 min.

RESULT

Activity = Δ OD/min x 62,000 units/ml.
Once the protein (neutrophil extract) has been determined, the activity may be reported as units/µg protein.

The lower limit of detection is taken as 0.2 units/µg protein.

APPENDIX 6

CALCULATION OF LINEAR REGRESSION AS USED FOR CREVICULAR ON BLOOD NBT POSITIVE VALUES IN CHAPTER 3

The purpose of this analysis is to obtain an idea of the extent to which one parameter is related to or depends upon another, but provides no information as to the reason for such a relationship. The correlation coefficient can thus be calculated and its statistical significance determined.

Let blood values = X
Let crevice values = Y
n = number of subjects and therefore values in each group

The regression is calculated from:

$$\Sigma x^2 = \Sigma X^2 - \frac{(\Sigma X)^2}{n} \qquad \Sigma y^2 = \Sigma Y^2 - \frac{(\Sigma Y)^2}{n}$$

$$\Sigma xy = \Sigma XY - \frac{\Sigma X . \Sigma Y}{n}$$

$$r = \text{correlation coefficient} = \frac{\Sigma xy}{\sqrt{\Sigma x^2 . \Sigma y^2}}$$

The significance level can be obtained from published tables, e.g. Snedecor, 1956: 174.

The 'best fit' line is determined:

$$b = \text{regression coefficient or slope} = \frac{\Sigma xy}{\Sigma x^2}$$

b was calculated for the male and female subjects, as well as the total group.

To determine the plot points

$Y = a + bX$, where a is the intercept on the vertical axis (when $X = 0$), and b is the regression coefficient.

$\bar{X}$ and $\bar{Y}$ are known,

$\therefore \bar{Y} = a + b\bar{X}$

$\therefore a = \bar{Y} - b\bar{X}$

The line can then be drawn between the two points,

$X = 0, Y = a; \quad \bar{X}, \bar{Y}$

APPENDIX 7

THE MANN-WHITNEY U TEST

This is a ranking test for the comparison of two groups of data of unequal size. The method employed below is that published by von Fraunhofer and Murray (1976).

n_1 = number of cases in the smaller group (20)
n_2 = number of cases in the larger group (30)

Each value is assigned a rank, i.e. smallest 1, etc. (For any duplicate values, the average is used.)

The value of U is then calculated. U = number of times that a score in the group with n_2 cases precedes a score in the group with n_1 cases in the ranking order.

$$U = n_1 n_2 + \frac{n_1(n_1 + 1)}{2} - R_1$$

$$U' = n_1 n_2 + \frac{n_2(n_2 + 1)}{2} - R_2$$

R_1 = sum of ranks in group R_1; R_2 = sum of ranks in group R_2.

The correct value for U is the smaller, with the value of the larger denoted as U'.

$$U = n_1 n_2 - U'$$

The method for determining statistical significance depends on the sample size. For n_2 greater than 20, the distribution approaches normal so that

$$\text{mean} = u_U = \frac{n_1 n_2}{2}$$

It is therefore possible to calculate Z (the normal deviate), according to the formula:

$$z = \frac{U - \frac{(n_1 n_2)}{2}}{\sqrt{\frac{(n_1)(n_2)(n_1 + n_2 + 1)}{12}}}$$

In the analysis for the two groups in chapter 4,

$$U = 126.5$$
$$U' = 473.5$$
$$\therefore U = 126.5$$
$$\therefore z = 3.4358, \text{ and } p \leq 0.0003.$$